The Significance of the OXA-51-like β-lactamases of *Acinetobacter baumannii*.

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I. Abstract.

The genus *Acinetobacter* currently contains 34 species, the vast majority of which are not regularly implicated in causing infection. However, incidences of hospital-acquired infection with *Acinetobacter* species are increasing, mainly due to the rise in the number of infections caused by the species *Acinetobacter baumannii* in immunocompromised patients particularly in intensive care units (ICUs). Due to high levels of resistance in *A. baumannii* to many classes of antibiotic, the carbapenems have been portrayed as the 'drugs of choice' for treating infections with this species. However, the activity of the carbapenems against *A. baumannii* has come under threat with the identification of four groups of class D β-lactamases carried by members of the species. Of these, the OXA-51-like enzymes have been suggested to be ubiquitous and intrinsic enzymes within *A. baumannii*. This presents the worrying scenario of the potential for all *A. baumannii* to become carbapenem resistant, leaving few treatment options available for this species. This project aimed to investigate the epidemiological spread of the OXA-51-like β-lactamases, examine the diversity within these enzymes, and whether this diversity may have implications for their ability to confer resistance to the carbapenems.

A functional map showing the amino-acid similarities between the OXA-51-like enzymes demonstrated that the enzymes fall into distinct closely-related groups, with notable clusters surrounding OXA-66, OXA-69 and OXA-98. PCR and sequencing analysis of a geographically diverse group of 64 *A. baumannii* isolates demonstrated that isolates forming specific sequence groups (SGs) as defined by Turton *et al* (2007) also contained the same or closely related $bla_{\text{OXA-51-like}}$ gene. Higher minimum

inhibitory concentrations (MICs) of carbapenems were found in association with acquired carbapenemases, or with the presence of IS*Aba1* upstream of the *bla*$_{\text{OXA-51-like}}$ gene. Pulsed-field gel electrophoresis (PFGE) analysis of the isolates did not demonstrate relatedness between isolates which formed the same sequence group. Multilocus sequence typing (MLST) of a subset of 44 isolates grouped isolates more consistently with the SGs and *bla*$_{\text{OXA-51-like}}$ alleles, indicating that PFGE is unreliable for use with *A. baumannii* unless studying a short time period, and that *bla*$_{\text{OXA-51-like}}$ alleles are a good epidemiological marker.

Mutation studies using meropenem with five *A. baumannii* isolates encoding different OXA-51-like enzymes, while resulting in an increase in meropenem MICs of between 8- and 128-fold, did not result in a nucleotide substitution in the *bla*$_{\text{OXA-51-like}}$ genes or a change in the upstream region of the genes in any isolate suggesting that the carbapenems may not be producing a strong selective pressure on the *bla*$_{\text{OXA-51-like}}$ genes. Analysis of $\pi N/\pi S$ ratios for the *bla*$_{\text{OXA-51-like}}$ genes, MLST genes and the TEM, SHV and CTX-M β-lactamase families showed the *bla*$_{\text{OXA-51-like}}$ genes to be under less positive selection than these other β-lactamases, though under less purifying selection than the MLST genes. Phylogenetic analysis of the MLST genes and the *bla*$_{\text{OXA-51-like}}$ genes indicates that the *bla*$_{\text{OXA-51-like}}$ genes have been evolving within *A. baumannii* since its speciation, and that different groups of *bla*$_{\text{OXA-51-like}}$ genes have been evolving at different rates corresponding to different rates of evolution within their parent lineages.

Structural modelling studies based upon the published crystal structure for OXA-40 indicated that amino-acid variation at particular sites in the OXA-51-like enzymes are likely to have an effect of enzyme function. Alterations at amino-acid position 167 change the shape of the entrance to the active site which may affect hydrolysis by accommodating the antibiotic differently, or may affect the substrate profile of the enzyme. The substitution of glutamine for proline at position 194 may significantly alter the shape of the enzyme thereby affecting substrate hydrolysis.

This project found that specific groups of *bla*$_{\text{OXA-51-like}}$ genes are associated with specific *A. baumannii* lineages and that these genes could serve as convenient epidemiological markers. Most of the diversity within the OXA-51-like enzymes is due to their continued evolution within *A. baumannii* since the species' emergence. However, certain amino-acid changes may play a role in altering the rate of hydrolysis or substrate profile of these enzymes.

II. Declaration.

The experiments and composition of this thesis are the work of the author unless otherwise stated.

III. Acknowledgements.

Firstly I would like to acknowledge my supervisors Professor Sebastian Amyes and Dr. Ahmed Hamouda, whose advice and support has been invaluable for the completion of this study. I thank them for the faith they placed in me to pursue the areas of research that I wished to. I would also like to thank Dr. Kevin Towner, who as our collaborator not only provided me with isolates and experimental data, but with whom I have had many fruitful and stimulating discussions. I thank the other members of our group, who made things easier even when none of my experiments were working. Additionally I would like to thank Laura Emery, for her invaluable sequence analysis advice and for putting up with me while I wrote this thesis. Finally, I would like to thank the Medical Research Council for funding the studentship which allowed me to undertake this research.

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V. Publications and presentations.

1. B.A. Evans, A. Hamouda, K. J. Towner & S.G.B. Amyes. The Linkage of Specific *bla*$_{\text{OXA-51-like}}$ Genes in *Acinetobacter baumannii* to Common Multi-resistant Clones. Presented at the 47th ICAAC, 2007.

2. B. A. Evans, S. Brown, A. Hamouda, J. Findlay & S. G. B. Amyes. Eleven novel OXA-51-like enzymes from clinical isolates of *Acinetobacter baumannii*. *Clin Microbiol Infect* 2007; **13**; 1137-1138.

3. B. A. Evans, A. Hamouda, K. J. Towner & S. G. B. Amyes. OXA-51-like beta-lactamases and their association with particular epidemic lineages of *Acinetobacter baumannii*. *Clin Microbiol Infect* 2008; **14**; 268-275.

4. B. A. Evans, A. Hamouda & S. G. B. Amyes. OXA-type beta-lactamases in *Acinetobacter baumannii*: emerging from the shadow of the extended-spectrum β-lactamases. *Rev Med Microbiol* 2007; **18**; 63-72.

5. B. A. Evans, A. Hamouda, K. J. Towner & S. G. B. Amyes. The OXA-51-like Enzymes of *Acinetobacter baumannii*: Markers of Success? Presented at the 18th ECCMID, 2008.

6. B. A. Evans, A. Hamouda, K. J. Towner & S. G. B. Amyes. Typing and *bla*$_{\text{OXA-51-like}}$ genes of *Acinetobacter baumannii*. Presented at the 48th ICAAC, 2008.

7. A. A. Alsultan, A. Hamouda, B. A. Evans & S. G. B. Amyes. *Acinetobacter baumannii*: emergence of four strains with novel *bla*$_{\text{OXA-51-like}}$ genes in patients with diabetes mellitus. *J Chemother* 2009, **21**(3); 290-295.

8. B. A. Evans, A. Hamouda, K. J. Towner & S.G.B. Amyes. Novel genetic context of multiple *bla*$_{\text{OXA-58-like}}$ genes in *Acinetobacter* genospecies 3. Presented at the 19th ECCMID, 2009.

9. B. Evans, A. Hamouda, S.A. Abbasi, F.A. Khan & S.G.B. Amyes. High Prevalence of Unrelated Multidrug-resistant *Acinetobacter baumannii* Isolates in Pakistani Military Hospitals. Accepted for presentation at the 49th ICAAC, 2009.

10. K .J. Towner, B. Evans, A. Bertini, S. G. B. Amyes, A. Carattoli. Distribution of Intrinsic Plasmid *Rep* Genes and their Association with Class D OXA Carbapenemase Genes in European Isolates of *Acinetobacter baumannii*. Accepted for presentation at the 49th ICAAC, 2009.

VI. Abbreviations.

Å	Angstroms
ADC	*Acinetobacter*-derived cephalosporinases
ARDRA	Amplified ribosomal DNA restriction analysis
bp	Base pairs
BSA	Bovine serum albumin
BSAC	British Society for Antimicrobial Chemotherapy
CLSI	Clinical and Laboratory Standards Institute
cm	Centi-metres
DNA	Deoxyribonucleic acid
EC	European clone
ESAC	Extended-spectrum AmpC
ESBL	Extended-spectrum β-lactamase
ESI-MS	Electrospray-ionisation mass spectrometry
G+C	Percentage of DNA consisting of guanine and cytosine bases
I_A^s	Index of association standardised
ICU	Intensive care unit
IMP	Imipenem
kb	Kilo-base pairs
kDa	Kilo-Dalton
Km	Concentration of substrate at which the rate of enzyme reaction is half the maximum velocity
L	Litre
M	Molar
MER	Meropenem
mg	Milligram
MIC	Minimum inhibitory concentration

min	Minute
mL	Milli-litre
MLST	Multi-locus sequence typing
mM	Milli-molar
NCBI	National Center of Biotechnology Information
NCTC	National Collection of Type Cultures
OMP	Outer-membrane protein
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pH	Measure of acidity or basicity of a solution
phi test	Pairwise homoplasy index test
pmol	Pico-moles
RI	Resistance island
RND	Resistance-nodulation-division
rpm	Revolutions per minute
rRNA	Ribosomal-ribonucleic acid
s	Seconds
SE clone	South-east clone
SG	Sequence group
ST	Sequence type
TBE	Tris/borate/ethylenediaminetetraacetic acid
tRNA	Transfer-ribonucleic acid
U	Units
V	Volts
w/v	Weight by volume
µL	Micro-litre
µM	Micro-molar

πN	Number of nonsynonymous nucleotide substitutions
πS	Number of synonymous nucleotide substitutions
$\pi N/\pi S$	Ratio of nonsynonymous to synonymous nucleotide substitutions; indicates the nature of selection upon a site.

VII. Tables and Figures.

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1. Introduction:

1.1 The genus *Acinetobacter.*

1.1.1 The origin of the genus *Acinetobacter.*

The history of the genus *Acinetobacter* is a complicated one. It begins in 1911 with the description by Beijerinck of an organism isolated from a soil sample, which was named *Micrococcus calcoaceticus* (Henriksen, 1973). Between 1911 and 1957 a number of bacterial species were described, sometimes by different authors who assigned different names to the same organism; these included *Bacterium anitratum, Moraxella glucidolytica, Neisseria winogradsky, Alcaligenes haemolysans, Mima polymorpha,* and *Moraxella lwoffii* (Henriksen, 1973). The genus *Acinetobacter* was created in 1957 with the proposal that it should include all non-motile *Achromobacter* species. However, it wasn't until 1971 that taxonomists officially recognised the genus *Acinetobacter* following comparative biochemical studies by Baumann that demonstrated that all of these organisms belonged to the same genus, and the type species *Acinetobacter calcoaceticus* was proposed (Baumann *et al.*, 1968, Henriksen, 1973).

1.1.2 Current taxonomic status of the genus *Acinetobacter.*

Modern DNA-DNA hybridisation techniques and DNA sequencing analysis have facilitated the classification of the genus *Acinetobacter* and the species that it contains. The genus is a member of the order *Gammaproteobacteria* and belongs to the family *Moraxellaceae*. Phenotypically the genus *Acinetobacter* is defined as Gram-negative, strictly aerobic coccobacilli that are non-fermenting, catalase-

Acinetobacter species	Sources	Clinical relevance
A. calcoaceticus	Humans (clinical and non-clinical samples) and soil	+
A. baumannii	Humans (clinical and non-clinical samples) soil, meat and vegetables	++++
A. haemolyticus	Humans (clinical and non-clinical samples)	+
A. junii	Humans (clinical and non-clinical samples)	+
A. johnsonii	Humans (clinical and non-clinical samples) and animals	+
A. lwoffii	Humans (clinical and non-clinical samples) and animals	+
A. radioresistens	Humans (clinical and non-clinical samples), soil and cotton	+
A. ursingii	Humans (clinical and non-clinical samples)	+
A. schindleri	Humans (clinical and non-clinical samples)	+
A. parvus	Humans (clinical and non-clinical samples) and animals	+
A. baylyi	Activated sludge and soil	-
A. bouvetii	Activated sludge	-
A. towneri	Activated sludge	-
A. tandoii	Activated sludge	-
A. grimontii	Activated sludge	-
A. tjernbergiae	Activated sludge	-
A. generi	Activated sludge	-
A. venetianus	Seawater	-
A. beijerinckii	Humans (clinical and non-clinical samples), horses, soil, surface water	+
A. gyllenbergii	Humans (clinical samples)	+
genomic species 3	Humans (clinical and non-clinical samples), soil and vegetables	+++
genomic species 6	Humans (clinical and non-clinical samples)	+
genomic species 10	Humans (clinical and non-clinical samples), soil and vegetables	+
genomic species 11	Humans (clinical and non-clinical samples) and animals	+
genomic species 13BJ or 14TU	Humans (clinical and non-clinical samples)	+
genomic species 14BJ	Humans (clinical and non-clinical samples)	+
genomic species 15BJ	Humans (clinical and non-clinical samples)	+
genomic species 16	Humans (clinical and non-clinical samples) and vegetables	+
genomic species 17	Humans (clinical and non-clinical samples) and soil	+
genomic species 13TU	Humans (clinical and non-clinical samples)	+++
genomic species 15TU	Humans (clinical and non-clinical samples)	+
genomic species 'between 1 and 3'	Humans (clinical samples)	+
genomic species 'close to 13TU'	Humans (clinical samples)	+

Table 1: Species within the genus *Acinetobacter* (Dijkshoorn *et al*., 2007, Nemec *et al*., 2009).

positive, oxidase-negative, non-motile and non-fastidious, with a G+C DNA content of between 39% and 47% (Peleg *et al*., 2008). There are currently 33 species recognised within the genus, of which 20 have been named (Table 1) (Dijkshoorn *et al*., 2007, Nemec *et al*., 2009) . *Acinetobacter* species identification can be performed phenotypically for some species, and there are commercially available kits

such as the API 20 NE system that can be used for this purpose. However some of the species are very difficult to distinguish phenotypically. Four species – *A. calcoaceticus, A. baumannii, Acinetobacter* genomic species 3 and *Acinetobacter* genomic species 13TU – can only be distinguished using molecular techniques, and are often referred to as the *A. calcoaceticus-A. baumannii* complex (Peleg *et al*., 2008). A number of different methods have been proposed for discriminating between some or all of these species. These include tRNA spacer fingerprinting, sequencing of the 16S rDNA, *gyrB* and *rpoB* genes, PCR amplification of the *recA* gene, sequencing of the 16S-23S intergenic rDNA region, restriction analysis of the 16S rDNA gene (ARDRA), restriction analysis of the 16S-23S rDNA region, PCR amplification of the *gyrB* gene, and multilocus PCR followed by electrospray ionisation mass spectrometry (PCR/ESI-MS) (Ehrenstein *et al*., 1996, Ibrahim *et al*., 1997, Yamamoto *et al*., 1999, La Scola *et al*., 2006, Nowak and Kur, 1995, Chang *et al*., 2005, Vaneechoutte *et al*., 1995, Higgins *et al*., 2007, Dolzani *et al*., 1995, Ecker *et al*., 2006). However, there is no consensus on which methodology should be used. The variety of methods that are utilised by different laboratories can be problematic as methods vary in their specificity and sensitivity, making direct comparisons between data sets obtained following identification by different means less reliable.

1.1.3 The natural habitat of *Acinetobacter* species.

Most *Acinetobacter* species are generally isolated from environmental samples such as soil and sludge, or from non-clinical human samples such as skin swabs (Table 1). Many members of the genus are generally considered to be environmental organisms (Peleg *et al*., 2008). In a study of soil and water samples from California, USA, 28 of

30 soil samples and 29 of 30 water samples were culture-positive for members of the genus (Baumann, 1968). In a recent study of the microbial communities from the extreme environments of six Andean lakes, a total of five different acinetobacters were isolated, one of which was determined to be *A. johnsonii* (Ordonez *et al*., 2009). Members of the species *A. calcoaceticus* have been isolated from the soil surrounding plant roots and have been shown to promote plant growth *in vitro* (Peix *et al*., 2009), while *A. baylyi*, another organism isolated from the soil, has been shown to be able to acquire plant DNA through horizontal gene transfer (Pontiroli *et al*., 2009). An *A. baumannii* isolate has even been recovered from petroleum-contaminated desert soil in Kuwait, and has been shown to be able to degrade crude oil (Obuekwe *et al*., 2009).

A series of studies to examine the association of *Acinetobacter* species with humans have produced varied results. In a study of skin carriage in 192 healthy humans in London, UK, an *Acinetobacter* carriage rate of 44% was found, with *A. lwoffii* accounting for 61% of isolates, *Acinetobacter* genospecies 15BJ accounting for 12.5%, and *A. radioresistens* accounting for 8% (Berlau *et al*., 1999). A similar result was found in Cologne, Germany, where a study of 40 healthy people found an *Acinetobacter* carriage rate of 42.5%, with 58% of isolates accounted for by *A. lwoffii*, 20% by *A. johnsonii*, and 10% by *A. junii* (Seifert *et al*., 1997). However, a study of skin carriage by student nurses and medical students in Hong Kong found an *Acinetobacter* carriage rate of 37.8%, but that *Acinetobacter* genospecies 3 was the most prevalent accounting for 32% of isolates, with *Acinetobacter* genospecies 13TU the second most prevalent accounting for 12% (Chu *et al*., 1999). It is possible that

the difference in the skin flora observed in the study from Hong Kong may be due to the study group being in contact with hospitalised patients, where carriage of *Acinetobacter* species 3 and 13TU is more common.

While it is clear that the natural environment of many of the members of the genus *Acinetobacter* is aquatic or in soil, there is debate as to the natural habitat of a few species. In particular, *A. baumannii*, *Acinetobacter* genospecies 3 and *Acinetobacter* genospecies 13TU are generally not found in environmental samples, but rather in the hospital environment and in clinical samples.

1.2 *Acinetobacter* infections.

1.2.1 *Acinetobacter* community-acquired infections.

Some members of the genus *Acinetobacter* have risen to prominence relatively recently due to the increasing number of human infections that they are causing. While many members of the genus have been identified as causing infection, the majority of these species have only been associated with infection rarely, or indeed on only one recorded occasion. However, three species are implicated in causing infections far more regularly. These are *A. baumannii*, *Acinetobacter* genospecies 3 and *Acinetobacter* genospecies 13TU. In particular, infections with *A. baumannii* are a particular problem as they can be difficult to treat. *Acinetobacter* species are opportunistic pathogens, and generally only cause infections in people who are immunocompromised due to a previous underlying condition. In the community setting, reports of infections due to *Acinetobacter* species have been reported, though they are infrequent. A review of these reports found that in the majority of community cases patients were diagnosed with pneumonia, with most of the

remaining cases diagnosed as bacteraemia. The vast majority of patients were found to have underlying co-morbidities including chronic obstructive pulmonary disease, diabetes mellitus, renal disease, excess alcohol consumption and smoking. Infections were almost all reported to be due to *A. baumannii*; however due to the difficulties in species identification within the genus, and particularly between members of the *A. baumannii-A. calcoaceticus* complex, it is possible that other species were involved. Mortality associated with infections was high at 56%, though this is likely to be an over-estimation as mild cases of *Acinetobacter* infection will not have been reported. Interestingly the majority of the reports originated from areas with tropical or sub-tropical climates. Whether this is due to geographical and climatic, or social factors is not clear (Falagas *et al*., 2007).

1.2.2 *Acinetobacter* hospital-acquired infections.

While community-acquired *Acinetobacter* infections are rare, hospital-acquired infections are far more common, and of a greater concern. Infections are associated with immunocompromised patients, with infection rates often being highest in intensive care units (ICUs) and surgical wards. Commonly the organisms cause pneumonia, particularly associated with mechanical ventilation, and bloodstream infections following invasive procedures (Peleg *et al*., 2008). The three species that are regularly implicated in cases of hospital-acquired infection are *A. baumannii*, *Acinetobacter* genospecies 3 and *Acinetobacter* genospecies 13TU. Usually, epidemiological surveys have identified *A. baumannii* to be by far the most frequent cause of infection, though there are reports of higher rates of isolation of *Acinetobacter* genospecies 3 (Lim *et al*., 2007, Chen *et al*., 2007, Ecker *et al*., 2006,

Boo *et al*., 2009). While *Acinetobacter* genospecies 3 and 13TU tend to cause infections sporadically and are usually relatively susceptible to antimicrobials, the same cannot be said of *A. baumannii*. Crude mortality rates for *Acinetobacter* species other than *A. baumannii* have been found to be low, with values between 7% and 18% being reported (Choi *et al*., 2006, Seifert *et al*., 1994). However, crude mortality rates of between 8% and 43% have been reported for *A. baumannii* (Falagas *et al*., 2006). Additionally, the length of stay in hospital associated with *A. baumannii* infection was significantly longer than that for other *Acinetobacter* species (Choi *et al*., 2006). Outbreaks of *A. baumannii* are often clonal, and the spread of clones or lineages can be seen nationally and internationally. A large number of infections globally can be attributed to three of these epidemic lineages, that have been termed European clones I, II and III (Dijkshoorn *et al*., 1996, van Dessel *et al*., 2004). In the UK there is an ongoing infection control problem relating to the circulation of several clonal lineages of *A. baumannii*, termed the South-East clone (SE clone), OXA-23 clone I and OXA-23 clone II (Coelho *et al*., 2006b). The reason why some lineages of *A. baumannii* are particularly successful and spread internationally is unclear, but at least part of the answer is likely to lie in the relatively high level of multiple antimicrobial resistance, and the ability to acquire and maintain resistance determinants, that these lineages possess. It is for the reasons mentioned above that of all of the species belonging to the *Acinetobacter* genus, it is *A. baumannii* that is the organism that gives us the greatest cause for concern.

1.3 Antimicrobials and *A. baumannii*.

The rise in the number of infections caused by *A. baumannii* over the recent decades is of great concern due to the difficulties that are faced in administrating effective antimicrobial treatment. Intrinsic properties of the species such as chromosomally-encoded β-lactamases, an effective permeability barrier, and the ability to acquire and maintain resistance determinants on mobile genetic elements, have severely reduced the number of effective antibiotics that can be used against some isolates, in a few cases to zero (Valencia *et al*., 2009). These features have resulted in the elimination of the penicillins, cephalosporins, aminoglycosides, quinolones and tetracyclines as effective treatment options for many *A. baumannii* isolates. This has left the carbapenems as the only sustainable group of antibiotics to treat infections with *A. baumannii*, due to their good activity and low toxicity.

The carbapenems are a subgroup of the β-lactam antibiotics and have the broadest spectrum of activity within this group. The first carbapenem to be approved for use was imipenem in 1985, a stable derivative of thienamycin, discovered in the 1970s to be produced by *Streptomyces cattleya*. This was followed by meropenem in 1993, ertapenem in 2001, and most recently by doripenem in 2007 (Livermore, 2009). As with other β-lactams, the carbapenems bind to the penicillin-binding proteins in the bacterium, preventing the final transpeptidation of the peptidoglycan in the cell wall, ultimately resulting in the cell breaking down its own cell wall, causing cell death. The carbapenems are stable in the presence of most β-lactamases due to the presence of a trans-α-1-hydroxyethyl group at position 6, which protects the molecule from hydrolysis of the bond between positions 4 and 7 (Figure 1) (Zhanel *et al*., 2007).

However, in recent years there has been a worrying rise in the number of *A. baumannii* infections that are resistant to the carbapenems. The ability of *A. baumannii* isolates to resist the action of the carbapenems by various mechanisms described below leaves no obvious choice for treatment of infections with this organism.

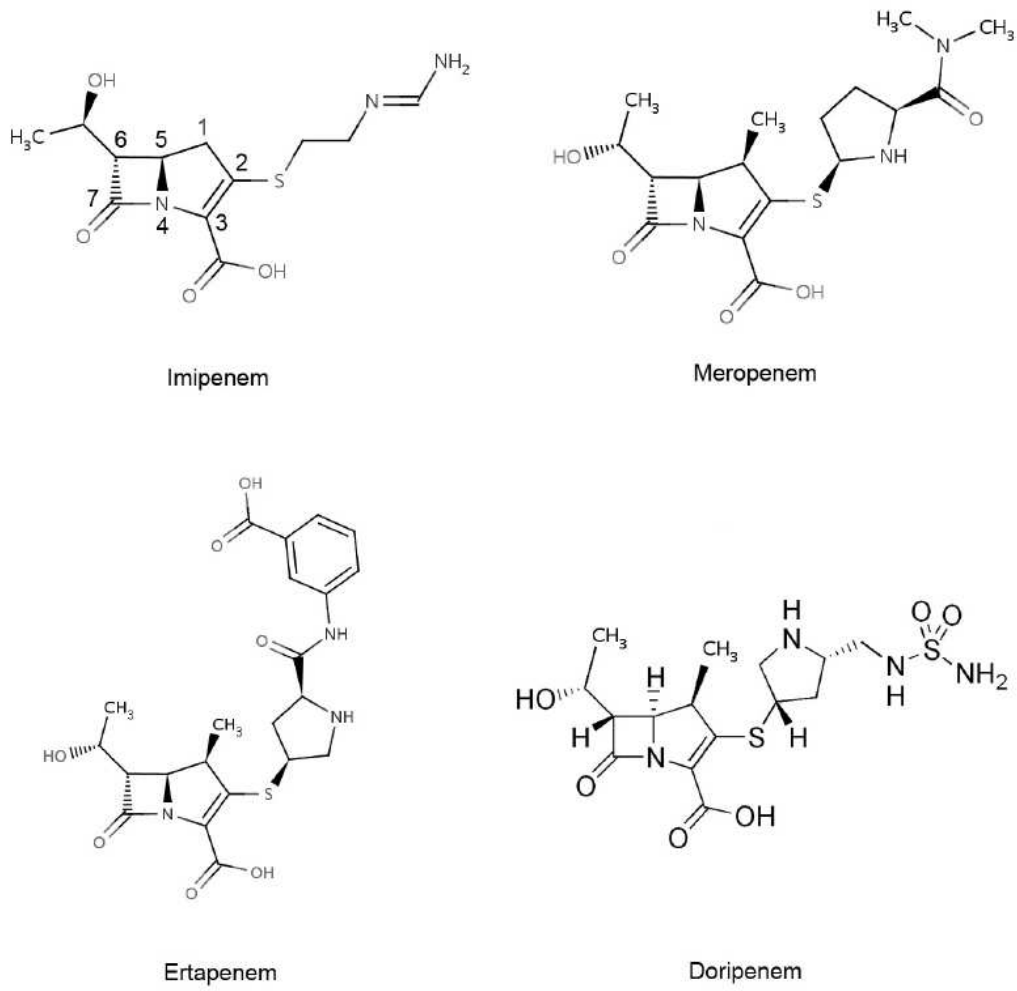

Figure 1: Chemical structures of the carbapenem β-lactams. The positions on the penem and β-lactam rings are numbered in the imipenem structure.

1.4 *A. baumannii* non-enzymatic β-lactam resistance mechanisms.

1.4.1 Penicillin-binding proteins.

Alterations to penicillin-binding proteins (PBPs) such that β-lactam antibiotics can no longer bind to them is a mechanism of resistance that is observed in many bacterial species (Zapun *et al*., 2008). In *A. baumannii* there have been few reports where PBPs have been examined. Where they have, it has been concluded that this resistance mechanism may be able to confer a low level of resistance to the carbapenems (Gehrlein *et al*., 1991, Fernandez-Cuenca *et al*., 2003). However, in these studies the β-lactam resistance observed could also be attributed to other enzymatic and non-enzymatic mechanisms of resistance (see below). Indeed, since the discovery of the enzymatic mechanisms of resistance described below, β-lactam resistance could be attributed to these mechanisms. Alteration of PBPs in *A. baumannii* is poorly studied, and it is likely that alterations in PBPs make a small but important contribution towards carbapenem resistance, particularly in *A. baumannii* isolates that lack an acquired carbapenemase. Further studies examining all the varied elements that may contribute to β-lactam resistance in *A. baumannii* would allow the role of the PBPs to be elucidated fully.

1.4.2 Outer membrane proteins.

A resistance mechanism that is found in many bacteria that can confer reduced susceptibilities to a number of antibiotic classes is the loss of outer membrane proteins (OMPs) that facilitate the transport of the antibiotic molecules across the cell membrane (Delcour, 2009). In *A. baumannii*, the loss of OMPs of several different sizes has been reported. Decreased expression of OMPs of sizes 33-36 kDa,

22 and 33 kDa, 29 kDa (subsequently named CarO), and 43 kDa (a protein with similarities to the important OprD OMP from *Pseudomonas aeruginosa*) have been observed in isolates that are resistant to the carbapenems (Clark, 1996, Bou *et al*., 2000a, Limansky *et al*., 2002, Dupont *et al*., 2005). Additionally, it has been demonstrated that a carbapenem-resistant *A. baumannii* isolate had a modified form of the CarO protein when compared to a sensitive isolate (Siroy *et al*., 2006). As with the PBPs, it is likely that reduced expression or modification of OMPs, while not conferring high levels of carbapenem resistance by themselves, make an important contribution in concert with other mechanisms to increased carbapenem resistance.

1.4.3 Efflux.

Efflux systems that actively pump antibiotics out of the cell are able to confer resistance to a wide range of antibiotic classes and are common amongst bacteria (Van Bambeke *et al*., 2000). There are a number of different families of related efflux pumps, but in *A. baumannii* the best studied of these belong to the resistance-nodulation-division (RND) family, in particular the AdeABC pump, and these have been found to be able to transport β-lactams out of the bacterium. There are three components of this pump, the *adeA*, *adeB* and *adeC* genes, which encode the membrane fusion component, the drug transporter component and the outer membrane porin respectively. Together with the two regulatory genes *adeR* and *adeS*, they are widespread in *A. baumannii* isolates particularly within the epidemic lineages, though *adeC* is less frequent as it is not thought to be an essential component for efflux (Nemec *et al*., 2007). The pump has been shown to contribute towards cefotaxime resistance, and to high levels of carbapenem resistance in

combination with acquired carbapenemases (Heritier *et al*., 2005b, Magnet *et al*., 2001).

1.5 *A. baumannii* enzymatic β-lactam resistance mechanisms.

1.5.1 Class A β-lactamases.

The Ambler class A β-lactamases hydrolyse variously the penicillins and the cephalosporins and are generally susceptible to β-lactamase inhibitors. This enzyme group includes the majority of the extended-spectrum β-lactamases (ESBLs) as it encompasses the TEM, SHV and CTX-M enzyme families. Class A β-lactamases appear to be reasonably widespread in *A. baumannii*. The gene encoding the enzyme PER-1 has been found in many isolates in the USA, Korea and Turkey (Hujer *et al*., 2006, Kim *et al*., 2008, Poirel *et al*., 2005a), and a large number of isolates from France and Belgium possess the gene for the VEB-1 enzyme (Naas *et al*., 2006b, Naas *et al*., 2006a). Isolates encoding TEM-, SHV- and CTX-M-type enzymes have also been described (Naas *et al*., 2007, Nagano *et al*., 2004, Hujer *et al*., 2006). Due to generally widespread high levels of resistance in *A. baumannii* to the penicillins and cephalosporins conferred by the intrinsic class C β-lactamases, these drugs are not usually considered appropriate for treatment of *A. baumannii* infections. As such, the prevalence of the class A β-lactamases in *A. baumannii* is not of major public health concern in itself; however the potential of this species to act as a reservoir for mobilisable resistance genes in the hospital may be of greater concern.

1.5.2 Class B β-lactamases.

The Ambler class B β-lactamases (often called the metallo-β-lactamases) differ from the class A, C and D enzymes in that they have one or two metal ions, usually zinc, in their active site rather than a serine residue. These enzymes are able to hydrolyse to various degrees all of the β-lactam antibiotics except for aztreonam, and are not inhibited by β-lactamase inhibitors (Walsh *et al*., 2005). Enzymes of the IMP and VIM families in particular are becoming more common in *A. baumannii* especially in areas such as the Far East, South America, and Greece (Jeong *et al*., 2006, Koh *et al*., 2007, Tsakris *et al*., 2008, Fritsche *et al*., 2005). The enzymes are usually carried on integrons, and this mobility combined with their ability to hydrolyse the carbapenems makes these enzymes a potential problem for successful antibiotic therapy. However, in *A. baumannii* the metallo-enzymes often appear to be expressed at low levels, and their presence can be difficult to detect phenotypically (Ikonomidis *et al*., 2008).

1.5.3 Class C β-lactamases.

The genes for Ambler Class C β-lactamases, also referred to as AmpC enzymes, are commonly found on the chromosome in Gram-negative organisms. As well as these intrinsic enzymes, there are several families of plasmid-borne *ampC* genes that are spreading particularly within the *Enterobacteriaceae* (Jacoby, 2009). In *Acinetobacter* species the class C enzymes are referred to as ADC enzymes, for *Acinetobacter*-derived cephalosporinases (Hujer *et al*., 2005). As this name implies, these enzymes are able to hydrolyse penicillins and the narrow-spectrum cephalosporins. When over-expressed, the enzymes can confer resistance to the

extended-spectrum cephalosporins, except for the fourth-generation cephalosporins. In other Gram-negative species, expression of the *ampC* gene is inducible, but in *A. baumannii* the genes are expressed when the insertion sequence IS*Aba1* inserts upstream of the gene where it provides a promoter for gene expression (Corvec *et al*., 2003). The widespread nature of the bla_{ADC} genes is the major reason for high levels of resistance in *A. baumannii* to the penicillins and cephalosporins and as such these drugs are generally not effective for treatment of this organism.

1.5.4 Class D β-lactamases.

1.5.4.1 The OXA-23-like enzymes.

The enzyme OXA-23, originally named ARI-1, was first identified in an *A. baumannii* strain from Scotland isolated in 1985 with an MIC for imipenem of 16 mg L^{-1} (Paton *et al*., 1993). The enzyme was shown to confer resistance to imipenem, and subsequent sequence analysis identified the enzyme as belonging to the Ambler class D β-lactamases (Donald *et al*., 2000). Two further enzymes with 99% amino-acid identity to OXA-23 have been identified in *A. baumannii* and named OXA-27 and OXA-49. OXA-27 was identified in an isolate from Singapore isolated between 1995 and 1997 (Afzal-Shah *et al*., 2001), and OXA-49 was associated with an isolate from China and the sequence published online in 2003 (accession number AY288523). Recently, five further enzymes named OXA-102, OXA-103, OXA-105, OXA-133 and OXA-134 have been identified in isolates of *Acinetobacter radioresistens* (Poirel *et al*., 2008, Mendes *et al*., 2009, Boo and Crowley, 2009). These eight enzymes constitute the OXA-23-like enzyme group (Table 2). Limited kinetic analyses of the OXA-23, OXA-27 and OXA-49 enzymes demonstrate they

Group	Group Members	Location	Host Species
OXA-23-like	OXA-23, OXA-27, OXA-49, OXA-102, OXA-103, OXA-105, OXA-133, OXA-134	C and P	*A. baumannii, A. junii, A. radioresistens, Acinetobacter* genomic species 3*, Proteus mirabilis, Acinetobacter* phenon 5*, Acinetobacter* phenon 6/ct 13TU, *Acinetobacter* genomic species 13TU*, Acinetobacter* genomic species 10/11, *A. lwoffii*
OXA-40-like	OXA-40, OXA-25, OXA-26, OXA-72	C and P	*A. baumannii, A. haemolyticus, Acinetobacter* genomic species 3, *A. baylyi, Pseudomonas aeruginosa*
OXA-51-like	OXA-51, OXA-64 to OXA-71, OXA-75 to OXA-80, OXA-82 to OXA-84, OXA-86 to OXA-95, OXA-98 to OXA-100, OXA-104, OXA-106 to OXA-113, OXA-115 to OXA-117, OXA-120 to OXA-127	C	*A. baumannii, Acinetobacter* genomic species 13TU
OXA-58-like	OXA-58, OXA-96, OXA-97	C and P	*A. baumannii, A. junii, Acinetobacter* genomic species 3, *Acinetobacter* genospecies 13TU, *Acinetobacter* phenon 6/ct 13TU, *Acinetobacter* genomic species 9, *Acinetobacter* genomic species 10, *A. calcoaceticus, A. radioresistens*

Table 2: Summary of the class D OXA-type β-lactamases found in *Acinetobacter* species (Poirel *et al*., 2008, De Champs *et al*., 2002, Villegas *et al*., 2007, Heritier *et al*., 2005b, Marque *et al*., 2005, Wang *et al*., 2007, Afzal-Shah *et al*., 2001, Bou *et al*., 2000b, Ruiz *et al*., 2007, Da Silva *et al*., 2004, Turton *et al*., 2006a, Merkier and Centron, 2006, Brown *et al*., 2005, Brown and Amyes, 2005, Vahaboglu *et al*., 2006, Tsakris *et al*., 2007, Evans *et al*., 2007, Heritier *et al*., 2005a, Koh *et al*., 2007, Naas *et al*., 2007, Bogaerts *et al*., 2006, Coelho *et al*., 2006b, van Dessel *et al*., 2004, Poirel *et al*., 2006, Coelho *et al*., 2006a, Lee *et al*., 2009, Sevillano *et al*., 2008, Boo and Crowley, 2009, Mendes *et al*., 2009b, Chu *et al*., 2009). C, chromosomal; P, plasmid.

hydrolyse aminopenicillins and oxacillin, and weakly hydrolyse oxyimino-cephalosporins and carbapenems (Paton *et al*., 1993, Afzal-Shah *et al*., 2001). However, differences were observed between OXA-23 and OXA-27, with OXA-23 having a higher activity against cephaloridine and oxacillin and less activity against carbapenems than OXA-27 (Afzal-Shah *et al*., 2001).

The OXA-23-like enzymes have been found in a variety of *Acinetobacter* species, as well as in isolates of *Proteus mirabilis*, and their genes can be chromosomally located or located on a variety of different plasmids (Table 2) (Bogaerts *et al*., 2006, Bonnet *et al*., 2002). The G+C content of the $bla_{\text{OXA-23-like}}$ genes is 37.6-37.9 % (Bergogne-Berezin and Towner, 1996), which is outside the 39-47% range for the *A. baumannii* genome, suggesting that these genes have evolved in another species. The recent identification of $bla_{\text{OXA-23}}$, $bla_{\text{OXA-102}}$, $bla_{\text{OXA-103,}}$ $bla_{\text{OXA-105,}}$ $bla_{\text{OXA-133}}$ and $bla_{\text{OXA-134}}$ genes on the chromosome of several *Acinetobacter radioresistens* isolates suggests that the OXA-23-like enzymes may have originated in that species (Poirel *et al*., 2008). Isolates encoding OXA-23 have been found worldwide and are frequently associated with outbreaks of hospital infection (Villegas *et al*., 2007, Zhou *et al*., 2007, Coelho *et al*., 2006b, Heritier *et al*., 2005b, Marque *et al*., 2005). Of particular concern is the recent increase in reports of carbapenem-resistant *A. baumannii* isolates encoding OXA-23 from South America and South-East Asia. A survey of 542 *A. baumannii* isolates from 10 institutions in Colombia found an average of 33.6% of isolates resistant to a carbapenem, and in a subset of 66 of these resistant isolates, 65 were positive by PCR for $bla_{\text{OXA-23}}$ (Villegas *et al*., 2007). In a teaching hospital in China rates of carbapenem resistance have increased from 5% in the

period 1993 to 2003 to greater than 50% in isolates from ICU wards and 20% in non-ICU isolates in 2004; and a subsequent study of 221 resistant isolates from 11 institutions, including 117 isolates from the same teaching hospital, found 97.7% were positive for a *bla*$_{\text{OXA-23-like}}$ gene (Wang *et al*., 2007).

In vivo the OXA-23 β-lactamase contributes towards resistance to amoxicillin, ticarcillin, meropenem and imipenem. When transformed into susceptible *bla*$_{\text{OXA-23}}$-negative recipient strains, the enzyme confers intermediate to low level resistance to carbapenems, with the higher of these values achieved in combination with over-expression of the RND-type AdeABC efflux pump. However, when a naturally-occurring plasmid containing the *bla*$_{\text{OXA-23}}$ gene is electro-transformed into the same recipient strains, moderate to high MICs are achieved of 16 to >32 mg L^{-1}, with the highest values again found in the recipient over-expressing the AdeABC efflux pump, indicating the involvement of other genetic factors associated with the *bla*$_{\text{OXA-23}}$ gene in conferring resistance (Heritier *et al*., 2005b).

1.5.4.2 The OXA-40-like enzymes

OXA-40, originally named OXA-24, was first identified in *A. baumannii* isolates responsible for an outbreak of infection in a hospital in Spain in 1997 (Bou *et al*., 2000b). Two further enzymes named OXA-25 and OXA-26, both with >99% amino-acid identity to OXA-40, were identified in isolates from Belgium and Spain isolated between 1995 and 1997 respectively (Afzal-Shah *et al*., 2001). A fourth enzyme also with >99% amino-acid identity to OXA-40, named OXA-72, was identified in an isolate from Thailand and the sequence published online in 2004 (accession number

AY739646). These four enzymes constitute the OXA-40-like enzyme group (Table 2).

Kinetic studies carried out on OXA-40, OXA-25 and OXA-26 demonstrate that these enzymes are capable of hydrolysing penicillins, have weak activity against carbapenems, and very weak activity against some cephalosporins (Heritier *et al*., 2003, Afzal-Shah *et al*., 2001, Bou *et al*., 2000b). All three enzymes show greater hydrolytic activity against imipenem than meropenem, with OXA-40 being the most active. The recent description of the crystal structure of OXA-40 provides insights into the substrate specificity of the enzyme (Figure 2) (Santillana *et al*., 2007). Analysis of the arrangement of the active site elements demonstrates similarities between OXA-40 and the structures of other non-*A. baumannii* class D oxacillinases, and therefore does not explain the different substrate specificity of increased carbapenem and reduced oxacillin hydrolysis of this enzyme. However two residues, Tyr-112 and Met-223, interact to form a hydrophobic tunnel that restricts access to the active site of the enzyme. Modelling predicts that molecules such as oxacillin, which has a large methylphenylisoxazolyl moiety, will therefore have their access to the active site reduced, while imipenem and meropenem with their small hydroxyethyl moieties are able to gain access more easily. Experiments on the OXA-40 β-lactamase with the Tyr-112 and Met-223 residues replaced with alanine demonstrated that the more enclosed hydrophobic environment surrounding the active site is also responsible for correctly orientating carbapenems for hydrolysis, which may help explain why the enzyme can hydrolyse carbapenems while class D oxacillinases cannot.

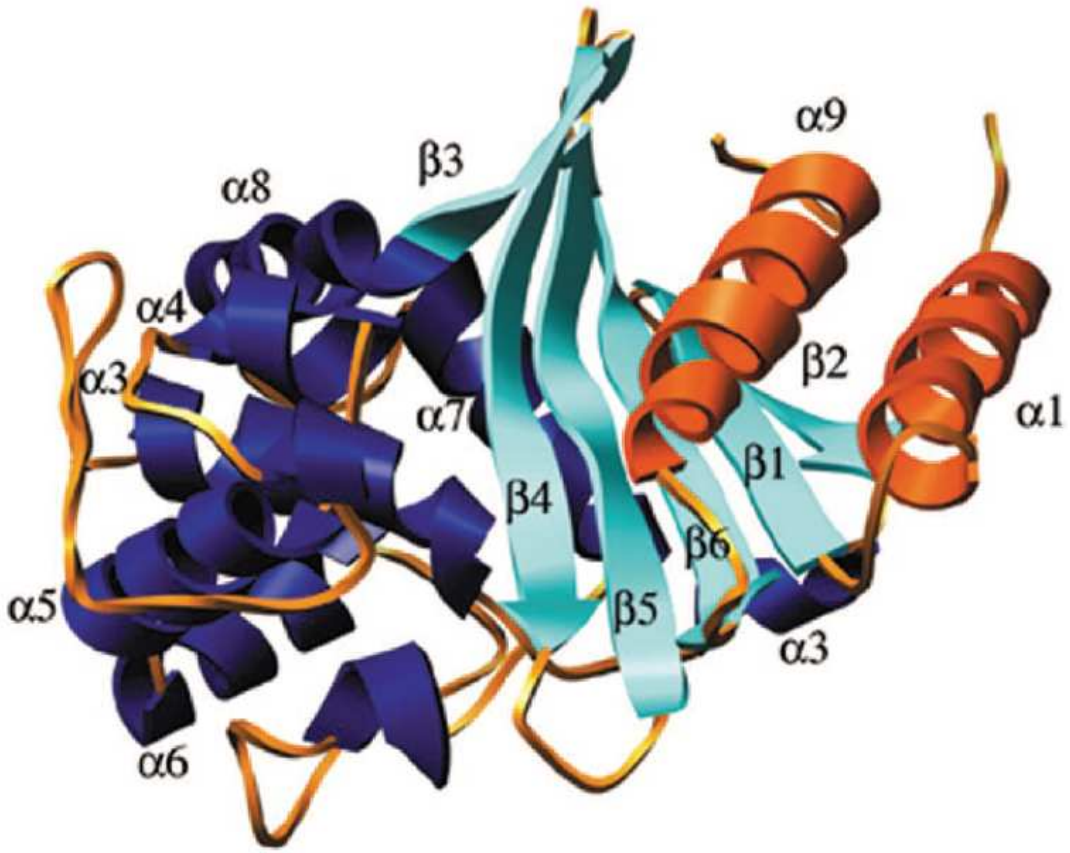

Figure 2: Structure of OXA-40 (modified from Santillana *et al*., 2007). The α-helix domain is shown in dark blue, the β-sheet of the mixed α/β domain is in cyan, and the α-helices are in red. The active site residues are found in the β4 strand, the α3 helix, and the loop between the α4 and α5 helices.

Despite only weak activity against the carbapenems of the purified enzyme, isolates encoding an OXA-40-like enzyme typically return MICs of >16 mg L^{-1} (Ruiz *et al*., 2007). A study by Heritier *et al* (2005b) demonstrated that *in vivo* the OXA-40 enzyme confers resistance to carbapenems as well as raising MICs to penicillins and cephalosporins. However, when transformed into susceptible reference *A. baumannii* strains the enzyme only confers intermediate to low level resistance, with the higher

of these values associated with an isolate over-expressing the AdeABC efflux pump, suggesting that a combination of mechanisms is required to achieve the high levels of resistance seen in clinical isolates.

The G+C content of the *bla*$_{\text{OXA-40-like}}$ genes is 33.9-34.3%, which differs from the content of the *A. baumannii* genome of 39-47%, suggesting that these genes originated in another species (Bergogne-Berezin and Towner, 1996). The *bla*$_{\text{OXA-40-like}}$ genes have been found in four species of *Acinetobacter* and in *Pseudomonas aeruginosa*, and to be both chromosomally and plasmid located (Table 2). While it has been found on a variety of different plasmids, the specific plasmid sizes have not been reported. Isolates encoding the enzymes have been reported in Asia (Wang *et al*., 2007, Mendes *et al*., 2009b, Lee *et al*., 2009), Belgium (Afzal-Shah *et al*., 2001) and the USA (Lolans *et al*., 2006), but the major geographic foci of the enzymes are Spain and Portugal (Ruiz *et al*., 2007, Da Silva *et al*., 2004, Quinteira *et al*., 2007, Sevillano *et al*., 2008). It is of concern that on the Iberian Peninsula there appears to be a high prevalence of the *bla*$_{\text{OXA-40-like}}$ genes. A recent study of 83 carbapenem-resistant *A. baumannii* isolates from 12 hospitals in Spain found 42% of isolates were positive for a *bla*$_{\text{OXA-40-like}}$ gene (Ruiz *et al*., 2007). A second recent study from a hospital in Portugal found that of 222 imipenem-resistant *A. baumannii* collected over the period of January 2001 to October 2004, 36.6% carried a *bla*$_{\text{OXA-40}}$ gene, the majority of which were associated with plasmid DNA, which was also found in *Acinetobacter haemolyticus* isolates in the same institution (Quinteira *et al*., 2007). The identification of a high percentage of *bla*$_{\text{OXA-40-like}}$ genes on plasmids presents the worrying possibility of these genes becoming more globally established.

1.5.4.3 The OXA-51-like enzymes

The OXA-51-like β-lactamases are an enzyme group that are intrinsic and ubiquitous in *A. baumannii* (Merkier and Centron, 2006, Turton *et al*., 2006b). The first enzyme of the group, OXA-51, was reported in 2004 in isolates obtained from Argentina in 1996 (Brown *et al*., 2005), and a large number of related enzymes have since been discovered. At the start of this project there were 32 enzymes making up the OXA-51-like group. The OXA-51-like enzymes now represent one of the largest groups of β-lactamases, with 60 enzymes currently identified (Table 2) (The Lahey Clinic, http://www.lahey.org/Studies/, accessed on 15th July 2009). The *bla*$_{\text{OXA-51-like}}$ genes have generally been found to be chromosomally located and non-transferable. However, in one report, PCRs for *bla*$_{\text{OXA-51-like}}$ genes using plasmid DNA as a template were positive, and the *bla*$_{\text{OXA-51-like}}$ gene could be cured from these isolates (Pournaras *et al*., 2006), and a second report mentions that the investigators were able to transfer a *bla*$_{\text{OXA-51-like}}$ gene to *Escherichia coli* (Vahaboglu *et al*., 2006), suggesting that it may be possible for these genes to be plasmid-borne. All *bla*$_{\text{OXA-51-like}}$ genes identified so far have been found in *A. baumannii* except for one, *bla*$_{\text{OXA-138}}$. This gene has been identified in an *Acinetobacter* genospecies 13TU isolate from Taiwan, further suggesting that there is the potential for these genes to be mobilised (Genbank accession number EU670845). However, the difficulties of species identification in the genus should be borne in mind when interpreting such data. The G+C content of the genes is 38.4-39.9%, which is similar to the content of the *A. baumannii* genome of 39-47%, lending further weight to the suggestion that these genes are native to *A. baumannii* (Bergogne-Berezin and Towner, 1996).

The contribution to resistance of these enzymes in *A. baumannii* has yet to be fully resolved. Kinetic analysis has only been performed on two enzymes – OXA-51 and OXA-69 (Brown *et al*., 2005, Heritier *et al*., 2005a). Both enzymes demonstrate poor hydrolysis of oxacillin and cloxacillin, a feature that has been observed in carbapenem-hydrolysing oxacillinases (Afzal-Shah *et al*., 2001, Bou *et al*., 2000b). High K_m values for imipenem and meropenem in OXA-69 indicate a poor affinity for these substrates, but OXA-51 has a low K_m for imipenem, suggesting a much higher affinity. However, OXA-69 weakly hydrolyses imipenem and meropenem, while OXA-51 weakly hydrolyses imipenem but not meropenem. The contribution of the enzymes to carbapenem resistance has been difficult to determine. Every member of the species possess a $bla_{\text{OXA-51-like}}$ gene, yet only a sub-set demonstrate carbapenem resistance. It has been suggested that the $bla_{\text{OXA-51-like}}$ genes are generally poorly expressed, and that they may only confer resistance when they have had their expression level increased with the insertion of a promoter on an IS*Aba1* insertion sequence element upstream of the gene (Turton *et al*., 2006a).

There is a large degree of diversity within the OXA-51-like enzymes, with members differing by up to 16 amino-acids. Within the OXA-51-like enzymes there are subgroups or clusters of enzymes that are associated with certain epidemic lineages (Evans *et al*., 2008). The large group of enzymes clustered around the OXA-66 β-lactamase are found in isolates belonging to an *A. baumannii* lineage including the prevalent European clone 2, while those clustered around the OXA-69 enzyme are found in another lineage encompassing European clone 1. The OXA-71 enzyme is associated with European clone 3. The most commonly identified enzymes are those

of the OXA-66 cluster, which are particularly highly represented in South America and Asia (Merkier and Centron, 2006, Wang *et al*., 2007, Koh *et al*., 2007, Evans *et al*., 2008). Enzymes of the OXA-69 cluster are also common, particularly in eastern Europe (Evans *et al*., 2008). Additionally OXA-71 is regularly identified and due to its association with European clone 3, is often identified in isolates from the Iberian Peninsula (van Dessel *et al*., 2004, Evans *et al*., 2008). It is unknown whether sequence variations in the OXA-51-like enzymes contribute to increased resistance or an altered spectrum resulting in certain enzymes being selected thus altering allele frequencies, or if certain enzymes are more prevalent than others.

1.5.4.4 The OXA-58-like enzymes

The enzyme OXA-58 was first identified in an *A. baumannii* clinical isolate in 2003 in France (Poirel *et al*., 2005b). The enzyme shares 48% and 49% amino-acid identity with OXA-23 and OXA-40 respectively, and was localised to a 30 kb plasmid. Two enzymes closely related to OXA-58 have since been identified. An *A. baumannii* isolated in 1996 from a hospital in Singapore contained a β-lactamase named OXA-96, which differs from the OXA-58 enzyme by a methionine substituting isoleucine at position 161 (Koh *et al*., 2007), and an enzyme named OXA-97 which differs from OXA-58 by a glycine substituting an alanine at position 53 has been identified in *A. baumannii* isolates in a hospital in Tunisia (Genbank accession number EF102240). These three enzymes constitute the OXA-58-like enzyme group (Table 2).

Kinetic analysis of OXA-58 has shown that it has similar properties to the other OXA-type carbapenemases of *A. baumannii*. The purified enzyme has weak activity against penicillins and imipenem, very weak activity against meropenem, and some activity against cephalothin and cefpirome, but not against ceftazidime or cefotaxime (Poirel *et al*., 2005b). However, OXA-58 hydrolyses imipenem twice as efficiently as OXA-40. When a plasmid carrying the $bla_{\text{OXA-58}}$ gene was transformed into two susceptible *A. baumannii* reference strains, it resulted in greatly increase MICs of amoxicillin and ticarcillin but only very small increases in carbapenem MICs, to which the strains remained susceptible. However, when a plasmid containing the $bla_{\text{OXA-58}}$ gene from a clinical isolate was transformed into the same reference strains, the MICs of carbapenems increased significantly in the strain over-expressing the AdeABC efflux pump (MICs of imipenem and meropenem of 32 mg L^{-1}). In the second strain, MICs were higher than when the strain was transformed with the artificial OXA-58 plasmid, though the strain remained sensitive (MICs = 2 mg L^{-1}) (Heritier *et al*., 2005b). This demonstrates that isolates carrying a $bla_{\text{OXA-58-like}}$ gene may require a combination of factors to achieve high level carbapenem resistance.

OXA-58-like enzymes have been found in a range of *Acinetobacter* species and are usually plasmid borne, though chromosomal location has been described (Bogaerts *et al*., 2006, Poirel *et al*., 2006). The G+C content of the $bla_{\text{OXA-58-like}}$ genes is 37.4-37.5%, which is slightly lower than the *A. baumannii* genome content of 39-47% (Bergogne-Berezin and Towner, 1996), indicating that these genes may have originated in another species. Isolates carrying $bla_{\text{OXA-58-like}}$ genes are most frequently reported from Europe, though the genes have been reported in isolates from South

America, North America, Asia Minor, Asia and Australia (Marque *et al*., 2005, Peleg *et al*., 2006, Coelho *et al*., 2006a, Koh *et al*., 2007, Wang *et al*., 2007, Lee *et al*., 2009, Mendes *et al*., 2009b, Zarrilli *et al*., 2008, Castanheira *et al*., 2008).

1.6 The role of mobile elements in resistance.

1.6.1 Insertion sequences.

1.6.1.1 IS*Aba1*.

The insertion sequence IS*Aba1* was first described in *A. baumannii* isolates from 2001, where it was found upstream of genes encoding the chromosomal class C β-lactamases, the ADC enzymes (Corvec *et al*., 2003). However, it was noted that sequences with identity to this new insertion sequence had been identified upstream of the $phaB_{AC}$ gene encoding an acetoacetyl-CoA reductase, as well as upstream of the original bla_{ARI-1} gene (now bla_{OXA-23}) in *A. baumannii* isolates previously. The element encodes two over-lapping open reading frames which, following a frame-shift during translation, form a functional transposase that is responsible for the transposition activity of the element (Mugnier *et al*., 2009). It has been demonstrated that when IS*Aba1* is found 9 base-pairs upstream of the bla_{ADC} gene in the opposite orientation to the β-lactamase gene it provides a promoter located between the transposase coding region and the inverted-repeat left that is responsible for increasing the expression of the bla_{ADC} gene (Heritier *et al*., 2006).

It has been suggested that the insertion of IS*Aba1* upstream of the bla_{OXA-23} gene increases the level of bla_{OXA-23} expression in a similar manner to the role it plays in increasing bla_{ADC} expression, as the insertion sequence has been found upstream of

bla$_{OXA-23}$ in >97% of carbapenem-resistant isolates (Zhou *et al*., 2007, Turton *et al*., 2006a, Heritier *et al*., 2006). Recent studies have shown that IS*Aba1* inserted 25 bp upstream of the *bla*$_{OXA-23}$ gene contains sequences which can act as promoters for its expression (Corvec *et al*., 2007, Segal *et al*., 2007). As well as potentially increasing expression, the insertion sequence may explain the widespread prevalence of *bla*$_{OXA-23}$ genes both in *A. baumannii* and in other *Acinetobacter* species as it can a form putative transposon named Tn*2006* (Corvec *et al*., 2007). As shown in Figure 3, the *bla*$_{OXA-23}$ gene can be flanked by two oppositely orientated IS*Aba1* sequences. The nine base-pair target site duplications after the inverted-repeat right of the upstream insertion sequence, and after the inverted-repeat right of the downstream insertion sequence, suggest that the whole structure can be mobilised through transposition events.

Similarly to its association with *bla*$_{ADC}$ and *bla*$_{OXA-23}$ genes, it has also been suggested that IS*Aba1* increases the expression of the *bla*$_{OXA-51-like}$ genes (Turton *et al*., 2006a). Comparisons of imipenem-resistant and imipenem-susceptible isolates from Taiwan demonstrated that the resistant isolates (MICs of imipenem and meropenem of 8 and 32 mg L^{-1} respectively to >32 mg L^{-1} for both antibiotics) had increased expression levels of their *bla*$_{OXA-51-like}$ genes relative to the susceptible isolates (MICs of imipenem and meropenem of 1 and 0.38 mg L^{-1} to 3 and 1.5 mg L^{-1} respectively), and that all of the resistant isolates were positive by PCR for an IS*Aba1* sequence 7bp upstream of the *bla*$_{OXA-51-like}$ gene while the sensitive isolates were not (Hu *et al*., 2007). Similar results were obtained in a study of Spanish isolates, where two isolates of the same pulsetype differed in *bla*$_{OXA-51-like}$ gene

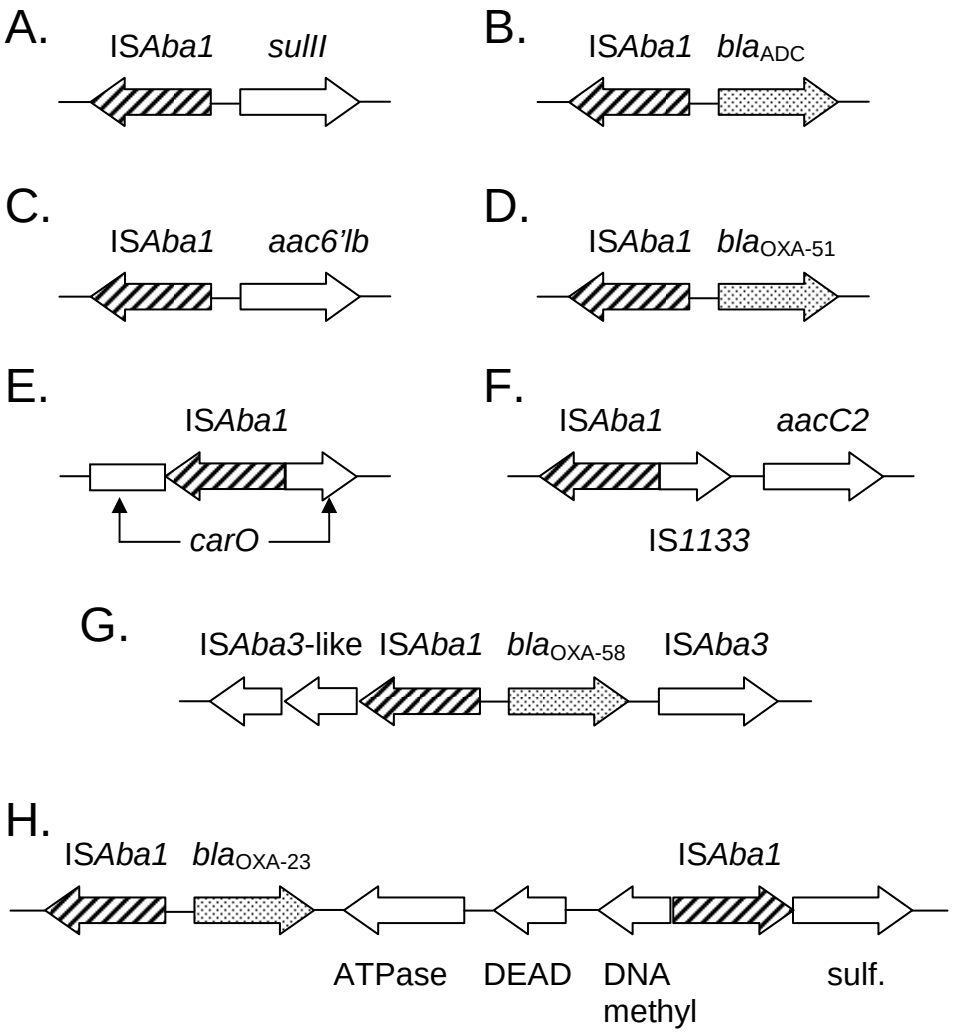

Figure 3: The interaction of IS*Aba1* with resistance determinants. A, IS*Aba1* twenty-one base-pairs upstream of the sulphonamide resistance gene *sulII* (Segal *et al*., 2005); B, the chromosomal class C β-lactamase of *A. baumannii* with IS*Aba1* nine base-pairs upstream (Heritier *et al*., 2006); C, IS*Aba1* upstream of the aminoglycoside-modifying enzyme *aac6'1b* (Naas *et al*., 2007); D; the intrinsic $bla_{\text{OXA-51-like}}$ β-lactamase of *A. baumannii* with IS*Aba1* seven base-pairs upstream (Turton *et al*., 2006a); E, the gene for the outer-membrane porin CarO is disrupted by an IS*Aba1* insertion (Lu *et al*., 2009); F, IS*Aba1* disrupts the insertion sequence IS*1133* upstream of the aminoglycoside-modifying enzyme *aacC2* (Segal *et al*., 2003); G, IS*Aba1* upstream of the $bla_{\text{OXA-58}}$ β-lactamase gene (Poirel and Nordmann, 2006); H, the composite transposon Tn*2006* potentially responsible for the mobilisation of the $bla_{\text{OXA-23}}$ β-lactamase gene (Corvec *et al*., 2007). DEAD, putative DEAD helicase; DNA methyl, DNA methylase; sulf, sulphonamide resistance gene.

expression with the isolate containing IS*Aba1* 7bp upstream of the gene having a higher level of expression than the isolate with IS*Aba1* elsewhere in the genome (Ruiz *et al*., 2007). Recently, the insertion of IS*Aba1* upstream of $bla_{\text{OXA-66}}$ was observed *in vivo*, and this resulted in a doubling of the MICs to imipenem (from 2 to 4 mg L^{-1}) and to meropenem (from 3 to 6 mg L^{-1}) (Figueiredo *et al*., 2009). Additionally, measurement of the expression levels of the $bla_{\text{OXA-66}}$ gene in the isolates demonstrated that the insertion of IS*Aba1* had resulted in a 50-fold increase in gene expression. However it is unclear whether up-regulation of the $bla_{\text{OXA-51-like}}$ gene alone can be sufficient or responsible for clinically significant levels of resistance to carbapenems in some isolates, and what the contribution may be of other resistance mechanisms working in concert, such as reduced outer membrane permeability (Hu *et al*., 2007).

IS*Aba1* has also been found in association with a variety of other genes involved in antimicrobial resistance (Figure 3). These include the sulphonamide resistance gene *sulII* (Segal *et al*., 2005), genes encoding aminoglycoside-modifying enzymes such as *aac6'1b* and *aacC2* (Naas *et al*., 2007, Segal *et al*., 2003), and the β-lactamase $bla_{\text{OXA-58}}$ (Poirel and Nordmann, 2006). The element has also been implicated in decreased membrane permeability through insertional inactivation of the gene encoding the pore-forming CarO protein (Lu *et al*., 2009), and in over-expression of the AdeABC efflux pump through insertional inactivation of the regulatory gene *adeS* (Ruzin *et al*., 2007).

1.6.1.2 IS*Aba3*.

The insertion sequence IS*Aba3* was first identified bracketing a *bla*$_{\text{OXA-58}}$ gene in an *A. baumannii* isolate from 2003 (Poirel *et al*., 2005b). The element contains a single open reading frame that encodes its transposase. IS*Aba3* has been found to be commonly associated with *bla*$_{\text{OXA-58}}$ genes (Poirel *et al*., 2006, Tsakris *et al*., 2008, Zarrilli *et al*., 2007). The element can be found upstream and in the opposite orientation to *bla*$_{\text{OXA-58}}$, where it is able to provide a promoter which is thought to increase the expression of the β-lactamase. The element is also commonly found downstream of *bla*$_{\text{OXA-58}}$, and it has been suggested that IS*Aba3* plays a role in the dissemination of this enzyme, though possibly through homologous recombination rather than through transposition (Poirel and Nordmann, 2006).

1.6.1.3 Other insertion sequences.

In addition to IS*Aba1* and IS*Aba3* which are commonly found in association with resistance determinants in *A. baumannii*, there are several other insertion sequences that have been identified in similar roles, though to be less abundant.

The insertion sequence IS*Aba4* has been found 25 bp upstream of *bla*$_{\text{OXA-23}}$ and to contain promoter sequences which again may play a role in increased gene expression (Corvec *et al*., 2007). Additionally, downstream of the *bla*$_{\text{OXA-23}}$ gene there is a truncated ATPase gene, followed by a sequence identical to the sequence at the end of the right inverted-repeat of IS*Aba4*. This was proposed as a potential transposon named Tn*2007*, and it was suggested that the transposon mobilised the *bla*$_{\text{OXA-23}}$ gene through a one-ended transposition mechanism (Corvec *et al*., 2007).

IS*Aba2* is an insertion sequence that is usually identified in association with *bla*$_{OXA-58}$ genes. The element can insert into the IS*Aba3* element that is found upstream of *bla*$_{OXA-58}$, in the same orientation as the β-lactamase gene. The formation of this chimeric element creates a hybrid promoter for *bla*$_{OXA-58}$ expression, with the -35 region being located within the right inverted repeat of IS*Aba2* and the -10 region located within the IS*Aba3* element (Poirel and Nordmann, 2006). The IS*Aba2*/IS*Aba3* chimeric element has been identified associated with *bla*$_{OXA-58}$ from different strains internationally and appears to be widespread (Zarrilli *et al*., 2007, Poirel *et al*., 2006, Bertini *et al*., 2007). Similarly, the insertion sequence IS*18* has been described as forming a chimeric element by inserting into IS*Aba3* and providing a hybrid promoter upstream of *bla*$_{OXA-58}$, though this element has been identified rarely (Poirel and Nordmann, 2006).

Insertion sequences in *A. baumannii* also appear to be involved in altering the copy number of the *bla*$_{OXA-58}$ gene, as duplication of the gene mediated by IS*26* elements has been observed. The study examined three clonal isolates, one containing one copy of *bla*$_{OXA-58}$, one containing two copies, and one containing three copies. The MICs of imipenem for the three isolates were 16, 32 and 128 mg L^{-1} respectively (Bertini *et al*., 2007). IS*26* appears to be able to duplicate the entire IS*Aba2*/IS*Aba3*-*bla*$_{OXA-58}$-IS*Aba3* region. This demonstrates that as well as increased expression due to promoters provided by insertion sequences, duplication of the *bla*$_{OXA-58}$ gene mediated by IS*26* elements can result in an increase in resistance to carbapenems.

1.6.2 Integrons.

Integrons are genetic elements that are able to capture and express multiple genes. They contain an integrase gene, *intI*, and an *attI* recombination site. Genes that are associated with an *attC* recombination site, called gene cassettes, that are recognised by the integrase can then be inserted into the integron. This can occur successively such that an integron carries multiple gene cassettes. Integrons are sorted into classes based upon the sequence of their integrase gene (Partridge *et al*., 2009). In *A. baumannii*, integrons are often identified with the majority being of the class 1 type. They commonly carry genes conferring resistance to the aminoglycosides, sulphonamides, chloramphenicol, trimethoprim and β-lactams (Ploy *et al*., 2000, Koeleman *et al*., 2001, Gombac *et al*., 2002, Kraniotaki *et al*., 2006, Fournier *et al*., 2006). While genes conferring resistance to the carbapenems of the bla_{IMP} and bla_{VIM} types have been found in *A. baumannii*, they are not generally widespread as yet, except in the Far East (Poirel and Nordmann, 2002). Enzymes of the class D OXA type that are able to confer carbapenem resistance have not been found on integrons.

1.6.3 Resistance Islands.

The term resistance island (RI) in bacteria refers to a region in a genome containing a high concentration of genes that encode resistance to antimicrobials that has been inserted into the bacterial chromosome. The first RI to be discovered in *A. baumannii* was published in 2006, and contained 45 resistance genes located within a ~96 kb region inserted into an ATPase gene (Fournier *et al*., 2006). Subsequently, five further variations of the RI have been identified, and it is thought that insertions of RIs of various sizes into the ATPase gene in *A. baumannii* is a relatively common

phenomenon (Iacono *et al*., 2008, Adams *et al*., 2008, Shaikh *et al*., 2009, Post and Hall, 2009). While these RIs carry genes conferring resistance to many classes of antibiotic, only the island named AbaR4 carries a gene that confers carbapenem resistance due to containing a *bla*$_{\text{OXA-23}}$ gene flanked by two IS*Aba1* sequences (Adams *et al*., 2008). The size and variability of these RIs demonstrate the prodigious ability that *A. baumannii* has to acquire and maintain resistance determinants, and therefore the problems that this bacterium poses and will continue to pose for the treatment of infections that it causes.

1.7 Summary.

- Only three species of *Acinetobacter* are regularly responsible for serious infection – *A. baumannii*, genospecies 3 and genospecies 13TU.

- *Acinetobacter* species cause serious infections in immunocompromised hosts.

- *A. baumannii* is a particular problem due to high levels of antimicrobial resistance encountered in this species.

- The carbapenems are regarded as the last sustainable group of antibiotics for treating infections caused by *A. baumannii* due to their high activity and low toxicity.

- Resistance to the carbapenems has been emerging in *A. baumannii*, mainly conferred by the class D OXA-type β-lactamases.

- In addition to acquired OXA-type β-lactamases, increased expression of the intrinsic $bla_{\text{OXA-51-like}}$ gene due to IS*Aba1* insertion upstream can lead to carbapenem resistance.

1.8 Aims of this project.

The recent emergence of carbapenem resistance in *A. baumannii*, the discovery of the *bla*$_{\text{OXA-51-like}}$ genes, and the observation that the presence of IS*Aba1* upstream of the *bla*$_{\text{OXA-51-like}}$ gene is associated with reduced susceptibility to the carbapenems led to the development of the following hypothesis that will be tested in this thesis:

- All *A. baumannii* possess a *bla*$_{\text{OXA-51-like}}$ gene, and could become resistant to the carbapenems through expression of this gene.

To test this hypothesis, a set of experiments were designed to answer a series of questions:

1. How prevalent are the *bla*$_{\text{OXA-51-like}}$ genes, and are all the different alleles distributed equally?
2. Are particular *bla*$_{\text{OXA-51-like}}$ alleles associated with particular *A. baumannii* strains, and are some strains more resistant to the carbapenems than others?
3. Can insertion sequences be found in association with all *bla*$_{\text{OXA-51-like}}$ alleles?
4. Do carbapenem-susceptible *A. baumannii* acquire resistance conferred by the *bla*$_{\text{OXA-51-like}}$ gene under selective pressure?
5. Do all of the OXA-51-like enzymes show structural similarities indicating a shared ability to hydrolyse similar substrates?

2. Materials and Methods.

2.1 Bacterial isolates.

Sixty-four *A. baumannii* isolates that were collected between 1982 and 2006 from hospitals worldwide were provided by Dr. Kevin Towner. Isolates were originally collected to monitor outbreaks of *A. baumannii* infection. Isolates had been initially identified in the individual hospital laboratories using standard microbiological techniques, and then confirmed as members of the *A. baumannii* species by tRNA fingerprinting (Ehrenstein *et al*., 1996). For identification experiments the type strain ATCC 19606 was used as a species 2 control strain, along with isolates Ab40, Ab41 and Ab191 previously identified as belonging to species 1, 3 and 13TU respectively. For minimum inhibitory concentration determination the control strains *Staphylococcus aereus* NCTC 6571, *Escherichia coli* NCTC 10418 and *Pseudomonas aeruginosa* NCTC 10662 were used, along with *A. baumannii* ATCC 19606. For amplification of bla_{OXA} genes, *A. baumannii* strains Ab33, Ab34 and Ab35, previously identified in our laboratory to be positive for $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-40-like}}$ and $bla_{\text{OXA-58-like}}$ genes respectively, were utilised. For $bla_{\text{OXA-51-like}}$ amplification, the control strain ATCC 19606 was included.

2.2 Identification.

Isolates that were found to be $bla_{\text{OXA-51-like}}$-negative by either the OXA multiplex PCR or the $bla_{\text{OXA-51-like}}$ whole gene PCR described below had their species identification confirmed by restriction analysis with endonucleases *Alu*I and *Nde*II of an amplified fragment of the 16S-23S intergenic rDNA sequence as described by Dolzani *et al* (1995). Following overnight culture at 37˚C on MacConkey agar plates,

one to two colonies of each isolate were inoculated into 5 mL of nutrient broth and incubated overnight at 37˚C and 180 rpm. DNA purification from these overnight liquid cultures was performed using a Puregene DNA Purification System Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA). PCR amplification of the 16S-23S intergenic rDNA sequence was performed using primer 1 (TTGTACACACCGCCCGTCA) and primer 2 (GGTACTTAGATGTTTCAGTTC) in 50 µL volumes containing 10 µL 5X Green GoTaq Flexi Buffer, 1.5 mM $MgCl_2$, 800 µM PCR nucleotide mix, and 1.25 U GoTaq DNA polymerase (Promega, Southampton, UK). Reactions were carried out in a Px2 Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) using primer concentrations of 25 pmol μL^{-1} and 0.5 µL template DNA. PCR conditions were as follows: 94°C for 5 mins, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, followed by 72°C for 5 mins. PCR products were analysed on 1.5% w/v agarose gels stained with ethidium bromide and scanned using the Diversity Database software image capturing system (Bio-Rad, Hemel Hempstead, UK).

Following successful amplification, PCR products were digested independently with *Alu*I and *Nde*II (Promega, Southampton, UK) at 37°C for 12 hours in 50µL volumes containing 23.5 µL distilled H_2O, 5 µL of restriction buffer, 0.5 µL bovine serum albumin, 20 µL of PCR product, and 1 µL of enzyme. Restricted products were then analysed an 4% w/v agarose gels stained with ethidium bromide and scanned using the Diversity Database software image capturing system (Bio-Rad, Hemel Hempstead, UK).

2.3 Minimum inhibitory concentration determination.

All isolates were tested for their susceptibility to imipenem and meropenem. Imipenem was utilised in the form of imipenem-cilastatin with a potency of 50% (Merck, Sharp & Dohme Ltd, Hertfordshire, UK), and meropenem with a potency of 99.8% was obtained from AstraZeneca (Cheshire, UK). Following overnight culture on MacConkey agar plates, isolates were grown overnight at 37˚C and 180 rpm in 5 mL of Iso-sensitest broth. Minimum inhibitory concentrations (MICs) were determined by doubling dilutions in Iso-sensitest agar, according to the British Society for Antimicrobial Chemotherapy (BSAC) methodology (British Society for Antimicrobial Chemotherapy, 1991). The antibiotic concentration range used was 0.008 to 128 mg L^{-1}. All agars and broths used during this project were obtained from Oxoid Ltd, Basingstoke, UK. The results were interpreted according to the guidelines of the BSAC (Andrews, 2007). *Pseudomonas aeruginosa* NCTC 10662, *Escherichia coli* NCTC 10418 and *Staphylococcus aureus* NCTC 6571 were used as quality control strains.

To compare the difference between the MIC values of imipenem and meropenem for isolates with an acquired OXA-type carbapenemase gene and those with IS*Aba1* element upstream of their $bla_{\text{OXA-51-like}}$ gene, a Mann-Whitney U test was conducted in the SPSS version 13 software (SPSS Inc, Chicago, Illinois, USA).

2.4 Amplification of bla_{OXA} genes and insertion sequences.

Amplification of the bla_{OXA} genes and insertion sequences of the isolates was achieved using PCRs. All primers used are listed in Table 3. Detection of the four

Primer	Sequence
OXA-23 1[a]	GATCGGATTGGAGAACCAGA
OXA-23 2[a]	ATTTCTGACCGCATTTCCAT
OXA-24 1[a]	GGTTAGTTGGCCCCCTTAAA
OXA-24 2[a]	AGTTGAGCGAAAAGGGGATT
OXA-51 1[a]	TAATGCTTTGATCGGCCTTG
OXA-51 2[a]	TGGATTGCACTTCATCTTGG
OXA-58 1[a]	AAGTATTGGGGCTTGTGCTG
OXA-58 2[a]	CCCCTCTGCGCTCTACATAC
ISAba1 B[b]	CATGTAAACCAATGCTCACC
ISAba1 A[b]	GTGCTTTGCGCTCATCATGC
ISAba2 A[b]	AATCCGAGATAGAGCGGTTC
ISAba2 B[b]	TGACACATAACCTAGTGCAC
ISAba3 A[b]	CAATCAAATGTCCAACCTGC
ISAba3 B[b]	CGTTTACCCCAAACATAAGC
OXA-69 A[c]	CTAATAATTGATCTACTCAAG
OXA-69 B[c]	CCAGTGGATGGATGGATAGATTATC
preABprom+[d]	GACCTGCAAAGAAGCGCTGC

Table 3: Primers used for *bla*$_{OXA}$ and insertion sequence amplification. [a], Woodford *et al* (2006); [b], Poirel *et al* (2006); [c], Heritier *et al* (2005a); [d], Heritier *et al* (2006).

groups of *bla*$_{OXA}$ genes was undertaken using a multiplex PCR by the method of Woodford *et al* (2006). Expected product sizes were 501 bp (*bla*$_{OXA\text{-}23\text{-}like}$), 246 bp (*bla*$_{OXA\text{-}40\text{-}like}$), 353 bp (*bla*$_{OXA\text{-}51\text{-}like}$) and 599 bp (*bla*$_{OXA\text{-}58\text{-}like}$). Amplification of fragments of the insertion sequences IS*Aba1*, IS*Aba2* and IS*Aba3* was achieved using primers designed by Poirel *et al* (2006) with expected product sizes of 434 bp, 1134 bp and 403 bp respectively. Following overnight culture at 37˚C on MacConkey agar plates, DNA extraction was performed by boiling up to three colonies in 50 µL sterile distilled water for 10 min. PCRs were performed in 50 µL volumes containing 10 µL 5X Green GoTaq Flexi Buffer, 1.5 mM $MgCl_2$, 800 µM

PCR nucleotide mix, and 1.25 U GoTaq DNA polymerase (Promega, Southampton, UK). All PCR conditions were as described by Woodford *et al* (2006). Reactions were performed under the following conditions: 94°C for 5 mins, then 30 cycles of 94°C for 25 s, 52°C for 40 s and 72°C for 50 s, followed by 72°C for 5 mins. Reactions were carried out in a Px2 Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) using primer concentrations of 25 pmol μL^{-1} and 0.5 µL template DNA. PCR products were analysed on 1.5% w/v agarose gels stained with ethidium bromide and scanned using the Diversity Database software image capturing system (Bio-Rad, Hemel Hempstead, UK).

To amplify the entire $bla_{\text{OXA-51-like}}$ gene the primers OXA-69A and OXA-69B designed by Heritier *et al* (2005a) which lie externally to the gene were used to amplify a 975 bp fragment in 50 µL volumes containing 20 mM Tris-HCL (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, nuclease-free BSA 0.1 mg mL^{-1}, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 800 µM PCR nucleotide mix, and 1.25 U of *Pfu* DNA polymerase (Promega, Southampton, UK). PCRs were performed under the following conditions: 95°C for 2 min, followed by 30 cycles of 95°C for 1 min, 48°C for 40 s and 72°C for 3 min, followed by 72°C for 6 min.

For isolates which produced a product larger than 975 bp because of the presence of IS*Aba1* upstream of the $bla_{\text{OXA-51-like}}$ gene, the primer preABprom+ (Heritier *et al*., 2006) was used with OXA-69B to produce a 1189 bp product under the same cycling conditions, except that the annealing temperature was increased to 53°C. Reactions and gel staining were carried out as above.

2.5 *bla*$_{\text{OXA-51-like}}$ gene sequencing.

PCR products generated using the primers OXA-69 A and B, or preABprom+ and OXA-69 B as described in section 2.4, were purified using a QIAquick PCR Purification Kit (Qiagen, Crawley, UK) and sequenced in both directions on a 3730 DNA Analyzer (Applied Biosystems, Warrington, UK). Sequencing reactions were performed using 5 µL of purified DNA template and 1 µL of primer at a concentration of 3.2 pmol μL^{-1}, and using the BigDye system. Sequencing was performed by technical staff at the Gene Pool, University of Edinburgh. Sequences were analysed using the BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and MultAlin (http://bioinfo.genopole-toulouse.prd.fr/multalin/) software. Sequences which appeared to be novel were re-amplified and re-sequenced to confirm this.

2.6 PCR walking.

In order to sequence and identify the *bla*$_{\text{OXA-51-like}}$ gene in isolates that failed to give a PCR product using primers OXA-69 A and B as described above, a genome walking technique using nested PCRs with specific and partially degenerate primers was employed. Reactions were performed as described by Guo and Xiong (2006) using the primers listed in table 4. PCRs were performed using GoTaq DNA polymerase and reaction components were the same as described in section 2.4. Primers OXA-51-OUT1 and -OUT2 were used in the initial touch-down PCR, which used cycling parameters of 95°C for 5 mins, followed by denaturing stages of 95°C for 45 s, annealing stages starting at 60°C and decreasing in a stepwise manner by 0.5°C per cycle, down to 47.5°C for 45 s, and elongation stages of 72°C for 2 mins. A final elongation of 72°C for 5 mins was performed at the end of the amplification cycle

block. Primers OXA-51-OUT3 and -OUT4 were used in the second stage amplification under the following conditions: 95°C for 5 mins, then 35 cycles of 95°C for 45 s, 50°C for 45 s, and 72°C for 2 mins, then a final elongation of 72°C for 5 mins. All specific primers were used in combination with the degenerate primers Semi-1, Semi-2 and Semi-3. PCR products were analysed, purified and sequenced as described in sections 2.4 and 2.5 above.

Primer	Sequence
Semi-1	GCCAATTCCGGATNGAYKSNGGNTC
Semi-2	GCCTTAAGGCCTANGARMSNCCNAG
Semi-3	CGGTTAAGGCCTANYTCSKNGANGC
OXA-51-OUT1	CAAGGCCGATCAAAGCATTA
OXA-51-OUT2	CCAAGATGAAGTGCAATCCA
OXA-51-OUT3	CTATATAAGGTGAGCAGGC
OXA-51-OUT4	GAATACCTAGCTCTGTTCG

Table 4: Primers used for walking out from the *bla*$_{\text{OXA-51-like}}$ genes.
Primers Semi-1,-2 and -3 were designed by Guo *et al* (2006). Degenerate primers contain non-standard bases with the following properties: N, any base; Y, pyrimidine; K, keto; S, strong; R, purine; M, amino.

2.7 Pulsed-field gel electrophoresis.

Isolates were typed by pulsed-field gel electrophoresis (PFGE) as described by Miranda *et al* (1996), with the following modifications. Cultures for making plugs were cultivated in 5 mL of nutrient broth. Plug slices were equilibrated in 100 µL of restriction endonuclease buffer for one hour at 4˚C prior to restriction with 30 units of *Apa*I restriction endonuclease overnight at 37˚C. Digested plug slices were run on

a 1% pulsed-field-certified agarose gel (Bio-Rad, Hertfordshire, UK) in 0.5 X TBE buffer using a pulse time of 5 to 35 seconds over 24 hours at 6 V cm^{-1}. A lambda ladder was used as a size standard (New England Biolabs, Hertfordshire, UK). Gels were run at 12˚C using CHEF DRII apparatus (Bio-Rad, Hertfordshire, UK), stained with ethidium bromide and scanned using the Diversity Database software image capturing system (Bio-Rad, Hertfordshire, UK). Gel analysis was performed using the BioNumerics v4.0 software (Applied Maths, Sint-Martins-Latem, Belgium). Banding pattern similarity was calculated using the un-weighted pair group method using arithmetic averages (UPGMA), with the Dice co-efficient at a tolerance of 1.3%.

2.8 Sequence group typing.

Two multiplex PCRs for the amplification of regions of the *ompA*, *csuE* and $bla_{\text{OXA-51-like}}$ genes for identifying sequence groupings were performed using consensus primers and amplification conditions as described by Turton *et al* (2007) and PCR components as described by Evans *et al* (2008). The sequences of the primers used in the multiplex PCRs are listed in table 5. PCRs were performed in 25 μL volumes using pre-formulated Ready-to-Go PCR beads (GE Healthcare Life Sciences, Little Chalfont, UK) containing PCR buffer, dNTPs and DNA polymerase. Primers were used at a final concentration of 400 nM. Reaction conditions were as follows: 94°C for 3 mins, then 30 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 1 min, then a final elongation stage of 72°C for 5 mins. PCR products were visualised as described in section 2.4.

Primers	Allele	Multiplex PCR	Sequence (5'-3')
Group1ompAF306	*ompA*	1	GATGGCGTAAATCGTGGTA
Group1and2ompAR660			CAACTTTAGCGATTTCTGG
Group1csuEF	*csuE*	1	CTTTAGCAAACATGACCTACC
Group1csuER			TACACCCGGGTTAATCGT
Gp1OXA66F89	*bla* $_{\text{OXA-51-like}}$	1	GCGCTTCAAAATCTGATGTA
Gp1OXA66R647			GCGTATATTTTGTTTCCATTC
Group2ompAF378	*ompA*	2	GACCTTTCTTATCACAACGA
Group1and2ompAR660			CAACTTTAGCGATTTCTGG
Group2csuEF	*csuE*	2	GGCGAACATGACCTATTT
Group2csuER			CTTCATGGCTCGTTGGTT
Gp2OXA69F169	*bla* $_{\text{OXA-51-like}}$	2	CATCAAGGTCAAACTCAA
Gp2OXA69R330			TAGCCTTTTTTCCCCATC

Table 5: Primers used for amplifying fragments for sequence group typing (Turton *et al*., 2007).

2.9 Multi-locus sequence typing.

Isolates were typed using a multi-locus sequence typing (MLST) scheme based upon that published by Bartual *et al* (2005) with modifications to the cycling parameters. Following overnight culture at 37˚C on MacConkey agar plates, one to two colonies of each isolate were inoculated into 5 mL of nutrient broth and incubated overnight at 37˚C and 180 rpm. DNA purification from these overnight liquid cultures was performed using a Puregene DNA Purification System Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA). PCRs for the seven 'housekeeping' genes *gltA, gyrB, gdhB, recA, cpn60, gpi* and *rpoD* were performed in 50 μL volumes containing 10 μL 5X Green GoTaq Flexi Buffer, 1.5 mM $MgCl_2$, 800 μM PCR nucleotide mix, 1.25 U GoTaq DNA polymerase (Promega, Southampton, UK), and 0.5 μL of DNA template. Primers described by Bartual *et al* (2005) were used at a final concentration of 0.25 μM under standard conditions with the annealing

temperatures described in Table 6. A Px2 Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) was used to conduct PCRs. Analysis of PCR products, product purification and sequencing were performed as described previously. Primers used for product amplification were also used for sequencing, except for in the case of the *gyrB*, *gdhB* and *rpoD* products. Primer pair M13 [-21] (TGTAAAACGACGGCCAGT) and M13 F (CAGGAAACAGCTATGACC) were used in sequencing *gyrB* fragments, primer pair GDH SEC F (ACCACATGCTTTGTTATG) and GDH SEC R (GTTGGCGTATGTTGTGC) were used in sequencing *gdhB* fragments, and primer pair 70FS (ACGACTGACCCGGTACGCATGTA) and 70RS (ATAGAAATAACCAGACGTAAGTT) were used in sequencing *rpoD* fragments. Resulting sequences were analysed using the online BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and MultAlin (http://bioinfo.genopole-toulouse.prd.fr/multalin/) software, and isolates were assigned to sequence types using tools on the *A. baumannii* MLST webpage (http://pubmlst.org/abaumannii/).

Concatenated sequences for all seven genes were used to estimate the phylogeny using the neighbour-joining method (Saitou & Nei, 1987) under the Jukes-Cantor genetic distance model (Jukes & Cantor, 1969), with statistical support for the nodes assessed via the bootstrap re-sampling method, with 1000 re-samples, implemented in Geneious v4.6.5. The phylogenetic tree was drawn using FigTree v.1.1.2 (http://tree.bio.ed.ac.uk/software/figtree/). Neighbour-joining trees for all identified alleles of the seven genes were constructed separately in the same manner as above.

gene	Primers	Primer Sequence (5' to 3')	Annealing
gltA	Citrato F1	AATTTACAGTGGCACATTAGGTCCC	55℃ for 1 min
	Citrato R12	GCAGAGATACCAGCAGAGATACACG	
gyrB	APRU F	TGTAAAACGACGGCCAGTGCNGGRTCYTTYTCYTGRCA	45℃ for 1 min
	UP1E R	CAGGAAACAGCTATGACCAYGSNGGNGGNAARTTYRA	
gdhB	GDHB 1F	GCTACTTTTATGCAACAGAGCC	55℃ for 1 min
	GDHB 775R	GTTGAGTTGGCGTATGTTGTGC	
recA	RA1	CCTGAATCTTCYGGTAAAAC	56℃ for 1 min
	RA2	GTTTCTGGGCTGCCAAACATTAC	
cpn60	CPN 3F2	ACTGTACTTGCTCAAGC	58℃ for 1 min
	CPN R2	TTCAGCGATGATAAGAAGTGG	
gpi	GPI F1	AATACCGTGGTGCTACGGG	58℃ for 1 min
	GPI R1	AACTTGATTTTCAGGAGC	
rpoD	70F RPOD	ACGACTGACCCGGTACGCATGTAYATGMGNGARATCGCNACNCT	45℃ for 1 min
	70R RPOD	ATAGAAATAACCAGACGTAAGTTNGCYTCNACCATYTGYTTYTT	

Table 6: PCR primers and modified conditions for amplification of the seven MLST genes. Primer sequences were designed by Bartual *et al* (2005). Degenerate primers contain non-standard bases with the following properties: N, any base; Y, pyrimidine; S, strong; R, purine; M, amino.

Comparisons of tree topologies were conducted visually, and differences confirmed using the quartet measure of tree-to-tree distance (Estabrook *et al*, 1985) implemented in the COMPONENT 2.0 software (http://taxonomy.zoology.gla.ac.uk/rod/cpw/index.html).

The START2 software (http://pubmlst.org/software/analysis/start2/) was also used to calculate the index of association standardised (I_A^S) for the seven loci (Haubold *et al*, 1998). Evolutionary relationships and clonal complexes within the isolates were investigated by eBURST analysis (Feil *et al*, 2004), using the software on the eBURST website (http://eburst.mlst.net/v3/enter_data/single/), with statistical support for the complexes assessed via the bootstrap re-sampling method, with 1000 re-samples. The pairwise homoplasy index test (phi test) was applied to investigate the possibility of recombination between the alleles within the seven 'housekeeping' genes (Bruen *et al.*, 2006), and was implemented in the SplitsTree4 software (http://www.splitstree.org/).

To investigate the degree of selection acting upon the seven MLST loci, $\pi N/\pi S$ ratios (Nei & Gojobori, 1986) were calculated in the START2 software and compared with those generated for $bla_{\text{OXA-51-like}}$, bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$ and bla_{CMY} genes, using the Kruskal-Wallis one-way analysis of variance implemented in the Minitab15 software (Minitab Ltd, Coventry, UK).

2.10 Dose response curves.

Following overnight culture on MacConkey agar plates at 37˚C, one colony for each isolate was inoculated into 10 mL nutrient broth and incubated overnight at 37˚C and 180 rpm. Volumes of 200 µL overnight broth culture were added to nutrient broths containing doubling dilutions of meropenem (AstraZeneca, Cheshire, UK) between 0.008 and 128 mg L^{-1} in a total volume of 9.8 mL. This was vortexed, then 100 µL of a 10^{-4} dilution was plated out onto a nutrient agar plate, to enable the number of cells at time = 0 hours to be determined. The antibiotic-containing cultures were incubated at 37˚C and 180 rpm for three hours, then 100 µL of 10^{-2}, 10^{-4} and 10^{-6} dilutions were plated out onto nutrient agar plates. All plates were incubated overnight at 37˚C, and colony counts were made for the initial time = 0 hours plates, and the appropriate dilution that gave a countable number of colonies for the time = 3 hours plates. This allowed the percentage viability after 3 hours of growth to be obtained.

2.11 Mutation study.

After overnight growth at 37˚C on MacConkey agar, one colony for each isolate studied was inoculated into 10 mL nutrient broth and incubated overnight at 37˚C and 180 rpm. Volumes of 100 µL of 10^{-5}, 10^{-6} and 10^{-7} dilutions of the overnight culture were plated out in triplicate onto nutrient agar plates, incubated overnight at 37˚C, and then the number of colonies were counted to calculate the viable count for that culture. Simultaneously, 100 µL volumes of culture were plated onto nutrient agar containing meropenem (AstraZeneca, Cheshire, UK) at 0.5X, 1X, 2X and 4X the meropenem MIC for that isolate, that had been previously determined. These plates were incubated at 37˚C for 48 hours, and then the numbers of colonies were

counted on all plates. One colony from the plate with the highest concentration of meropenem on which there was bacterial growth was inoculated into 10 mL of nutrient broth and incubated overnight at 37˚C and 180 rpm. Volumes of 100 µL of culture were plated out to determine viable counts as described above, and onto plates containing meropenem at 0.5X, 1X, 2X and 4X the concentration of meropenem from which the culture was derived. These plates were then incubated at 37˚C for 48 hours as before, and the whole process repeated until colonies were only being recovered at concentrations of meropenem equal to or less than that from which the culture was derived. These final stage mutants were screened for IS*Aba1* upstream of the $bla_{\text{OXA-51-like}}$ gene and had their $bla_{\text{OXA-51-like}}$ gene sequenced as described previously. The MICs for the parent and mutant isolates of imipenem and meropenem were determined. Freezer stocks of the mutant isolates were used that were frozen eight months prior to conducting MIC testing.

2.12 Growth study.

Following overnight culture on MacConkey agar, one colony of isolate A418 was used to inoculate 10 mL of nutrient broth, and 10 mL of nutrient broth containing 0.5 mg L^{-1} of imipenem (Merck, Sharp & Dohme Ltd, Hertfordshire, UK). Cultures were incubated for 24 hours at 37˚C and 180 rpm. Volumes of 100 µL of the cultures were then added to 9.9 mL nutrient broth or 9.9 mL nutrient broth containing 0.5 mg L^{-1} imipenem respectively and incubated for a further 24 hours. This process was repeated for 100 days. Freezer stocks of the cultures were made every 5 days by adding 900 µL of culture to 100 µL of glycerol and storing at -80˚C. The initial A418 isolate and the two 100 day-old isolates had their $bla_{\text{OXA-51-like}}$ gene amplified and

sequenced using primers OXA-69A-EXT (TGGATTAAATTTAGTTGCGAAGC) and OXA-69B-EXT (AATGGTTTCCCTGTCATTGG) as described in section 2.4, and were also typed by PFGE as described previously.

2.13 OXA-51-like functional map.

All publicly available OXA-51-like amino-acid sequences were obtained from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). Sequences were analysed using MultAlin software (http://bioinfo.genopole-toulouse.prd.fr/multalin/) and all amino-acid variations were recorded. OXA-69 was chosen as a starting point in constructing the map as this was the enzyme found in the oldest isolate in this study (A1 from 1982). The map was subsequently re-drawn with OXA-65 as a starting point (see Results). Branches were constructed for each enzyme by listing the amino-acid changes from OXA-65 from the most common across all enzymes first, to the least common last. The branches were drawn in order from the enzymes with the fewest differences from OXA-65 first, through to the enzymes with the highest number of differences. Branches with the same changes within them were merged to produce the fewest number of branches possible. Amino-acid changes were not reversed within the same branch.

2.14 *bla*$_{\text{OXA-51-like}}$ sequence analysis.

All available nucleotide sequences for *bla*$_{\text{OXA-51-like}}$ genes were downloaded from the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). The gene phylogeny was estimated using the bayesian method under the general time reversal model of nucleotide substitutions

with gamma-distributed rates, implemented in MrBayes (Huelsenbeck & Ronquist, 2001). The phylogenetic tree was drawn using FigTree v.1.1.2 (http://tree.bio.ed.ac.uk/software/figtree/).

To compare the diversity within the *bla*$_{\text{OXA-51-like}}$ genes and *A. baumannii* as a species, MLST data for the six available genome sequences (ACICU, AB0057, AB307-0294, AYE, SDF and ATCC17978) along with two *Acinetobacter* genospecies 13TU isolates (st11681 and st7961) was downloaded from the NCBI website and combined with MLST data generated in this project. These sequences were used to estimate a phylogeny for *A. baumannii* via the bayesian method as described previously.

2.15 OXA-51-like structural models.

Structural models of the OXA-51-like enzymes were created using the online protein homology/analogy recognition engine (phyre) (http://www.sbg.bio.ic.ac.uk/~phyre/), using the crystal structure for OXA-40 published by Santillana *et al* (2007) as a backbone. The OXA-40 structure was chosen for this purpose as it has the highest sequence homology to the OXA-51-like enzymes of all the published enzyme structures to date, at approximately 63% amino-acid identity (Peleg *et al*, 2008). Structures were viewed in the Swiss-PdbViewer DeepView version 4 (http://spdbv.vital-it.ch/). Structures were compared with one-another to identify amino-acid differences that may affect enzyme function. Additionally, to investigate the degree of selection acting upon certain regions of the OXA-51-like enzymes, $\pi N/\pi S$ ratios were calculated in the program DnaSP version 5

(http://www.ub.edu/dnasp/), within consecutive 50 amino-acid-long windows of an alignment of the enzyme sequences, with each window shifting 10 amino-acids down the sequence from the previous window. This was plotted against the percentage amino-acid difference of OXA-51-like sequences to an OXA-51-like consensus sequence to identify regions within the enzymes that may be under positive selection.

3. Results.

3.1 Characteristics of isolates analysed by sequence groups.

3.1.1 Sequence group 1 isolates.

Isolates were assigned to sequence groups (SGs) by the method of Turton *et al* (2007) by multiplex PCR performed by Dr. Kevin Towner. Sequence group 1 (SG1) formed the largest group, accounting for 44% of isolates. The second largest group was SG2, containing 28% of the isolates, and SG3 was the third largest group, representing 9% of the isolates. Representatives of the three European lineages were found in separate sequence groups, with European Clone 1 (EC1) belonging to SG2, European Clone 2 (EC2) belonging to SG1, and European Clone 3 (EC3) belonging to SG3, as described previously (Turton *et al*., 2007). Twelve (19%) isolates did not belong to one of the three major sequence groups, and produced novel combinations of products in the two multiplex PCRs.

Table 7 shows the isolates assigned to SG1, the *bla*$_{\text{OXA-51-like}}$ gene they possess, their carbapenem MICs and PCR screening results. There is no particular geographical focus for SG1 isolates, with isolates originating from countries across Europe as well as from Asia, Africa and South America. Of the 28 isolates, 22 (79%) possess the *bla*$_{\text{OXA-66}}$ gene. The remaining six isolates carry one of five genes that are similar to *bla*$_{\text{OXA-66}}$, differing by a maximum of three nucleotides (translating to one amino-acid) from this gene, except for *bla*$_{\text{OXA-108}}$, which is slightly farther removed and differs by seven nucleotides (translating to five amino-acids). Two of these genes, *bla*$_{\text{OXA-108}}$ and *bla*$_{\text{OXA-109}}$ were novel discoveries (for sequence alignments, see

Isolate	Origin	SG	*bla* OXA-51-like allele	IS*Aba1* upstream of *bla* OXA-51	*bla* OXA-23-like allele	*bla* OXA-40-like allele	*bla* OXA-58-like allele	IS*Aba1*	IS*Aba2*	IS*Aba3*	MICs (mg/L)	
											IMP	MER
A369	Oviedo, Spain	1	OXA-66	-	-	+	-	+	+	-	>128	>128
A332	Bilbao, Spain	1	OXA-66	-	-	+	-	+	+	-	64	128
A401	Taiwan	1	OXA-82	+	-	-	-	+	-	-	16	16
A380	London, United Kingdom	1	OXA-66	-	-	-	+	+	+	+	16	16
A392	Freiburg, Germany	1	OXA-66	-	-	-	+	+	+	+	8	8
A473	Warsaw, Poland	1	OXA-108	+	-	-	-	+	+	-	8	4
A371	Prague, Czech Republic	1	OXA-83	+	-	-	-	+	-	-	8	32
A484	Nottingham, United Kingdom	1	OXA-83	+	-	-	-	+	-	-	8	64
A387	Ioannina, Greece	1	OXA-66	-	-	-	+	+	+	+	4	4
A397	Thessalonika, Greece	1	OXA-66	-	-	-	+	-	+	+	4	4
A474	Warsaw, Poland	1	OXA-66	-	-	-	-	+	-	-	4	2
A418	Wroclaw, Poland	1	OXA-66	-	-	-	-	+	-	-	4	8
A343	Chesterfield, United Kingdom	1	OXA-109	+	-	-	-	+	-	-	2	4
A4	Nottingham United Kingdom	1	OXA-66	-	-	-	-	+	-	-	0.5	0.5
A47	Berlin, Germany	1	OXA-66	-	-	-	-	+	-	-	0.5	1
A365	London, United Kingdom**	1	OXA-66	-	-	-	-	+	-	-	0.5	0.5
A480	Nottingham, United Kingdom	1	OXA-66	-	-	-	-	+	-	-	0.5	0.5
A481	Nottingham, United Kingdom	1	OXA-66	-	-	-	-	+	-	-	0.5	2
A482	Nottingham, United Kingdom	1	OXA-66	-	-	-	-	+	-	-	0.5	1
A216	Sheffield, United Kingdom	1	OXA-66	-	-	-	-	+	-	-	0.5	1
A25	Nottingham, United Kingdom	1	OXA-66	-	-	-	-	+	-	-	0.25	0.5
A6	Nottingham, United Kingdom	1	OXA-66	-	-	-	-	+	-	-	0.25	0.5
A230	Nottingham, United Kingdom	1	OXA-66	-	-	-	-	-	-	-	0.25	1
A60	Buenos Aires, Argentina	1	OXA-65	-	-	-	-	+	-	-	0.25	1
A186	Johannesburg, South Africa	1	OXA-66	-	-	-	-	+	-	-	0.25	0.5
A140	Cologne, Germany	1	OXA-66	-	-	-	-	-	-	-	0.25	0.5
A24	Nottingham, United Kingdom	1	OXA-66	-	-	-	-	+	-	-	0.12	0.25
A320	RUH 134 European clone II	1	OXA-66	-	-	-	-	+	-	-	0.12	0.5

Table 7: Isolates assigned to SG1. IMP, imipenem; MER, meropenem; SG, sequence group. **Isolate belonging to the South East clone.

appendix A). None of the isolates carry a *bla*$_{\text{OXA-23-like}}$ gene. Only two isolates, A369 and A332 both from Spain, possess a *bla*$_{\text{OXA-40-like}}$ gene, and these two isolates also have the highest levels of resistance to both imipenem and meropenem. A *bla*$_{\text{OXA-58-like}}$ gene was detected in four isolates, and these isolates also had intermediate to high levels of carbapenem resistance. IS*Aba1* was found in 25 isolates (89%). However it was only located upstream of the *bla*$_{\text{OXA-51-like}}$ gene in five isolates, and all five of these isolates possessed a *bla*$_{\text{OXA-66-like}}$ gene rather than *bla*$_{\text{OXA-66}}$ itself. These isolates all had intermediate to high levels of resistance to the carbapenems. IS*Aba2* was amplified from seven isolates, all but one of which also harboured either a *bla*$_{\text{OXA-40-like}}$ or *bla*$_{\text{OXA-58-like}}$ gene. IS*Aba3* was located in just four isolates, and were the same four isolates that possessed a *bla*$_{\text{OXA-58-like}}$ gene.

An example gel showing results of the multiplex PCR screening for the presence of *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-40-like}}$, *bla*$_{\text{OXA-51-like}}$ and *bla*$_{\text{OXA-58-like}}$ genes is shown in figure 4. An example gel showing results of the PCR amplification of the entire *bla*$_{\text{OXA-51-like}}$ gene, also indicating the presence of IS*Aba1* upstream of the gene is shown in figure 5. Examples of the results of PCR screening for IS*Aba1*, IS*Aba2* and IS*Aba3* are shown in figures 6, 7 and 8 respectively.

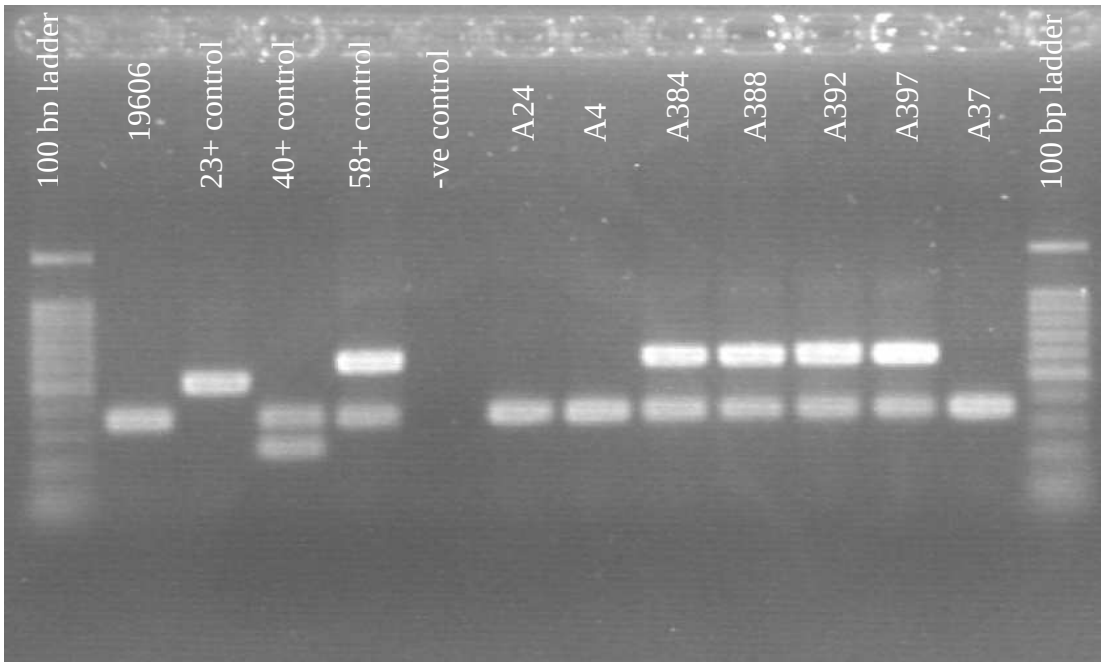

Figure 4: Example gel for the OXA multiplex PCR. Lanes are labelled with markers, controls or isolate names.

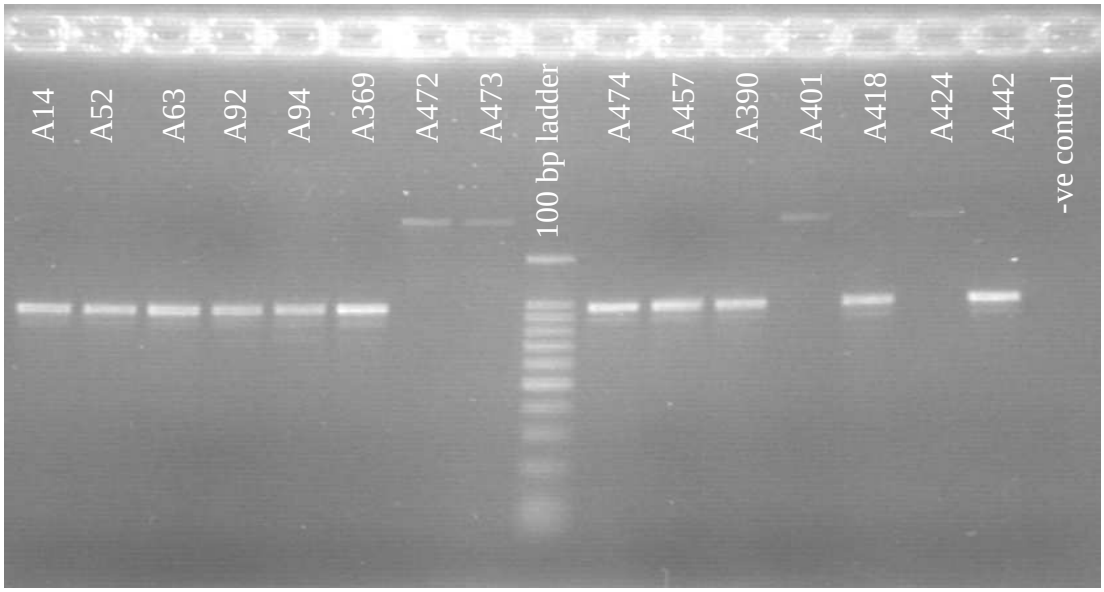

Figure 5: Example gel for the amplification of the entire *bla*$_{\text{OXA-51-like}}$ gene. Larger band sizes represent the presence of IS*Aba1* upstream of the *bla*$_{\text{OXA-51-like}}$ gene. Lanes are labelled with markers, controls or isolate names.

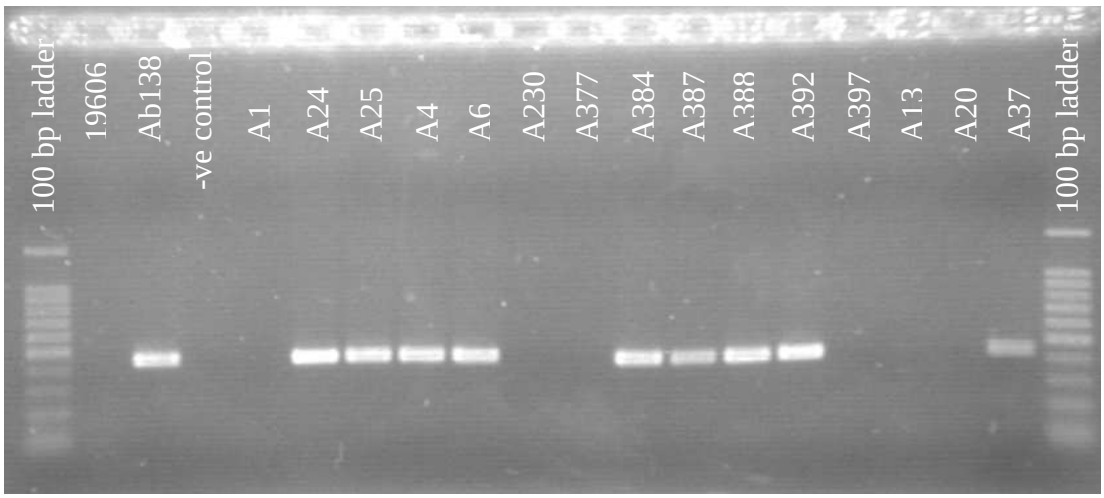

Figure 6: Example gel for the IS*Aba1* PCR. Lanes are labelled with markers, controls or isolate names.

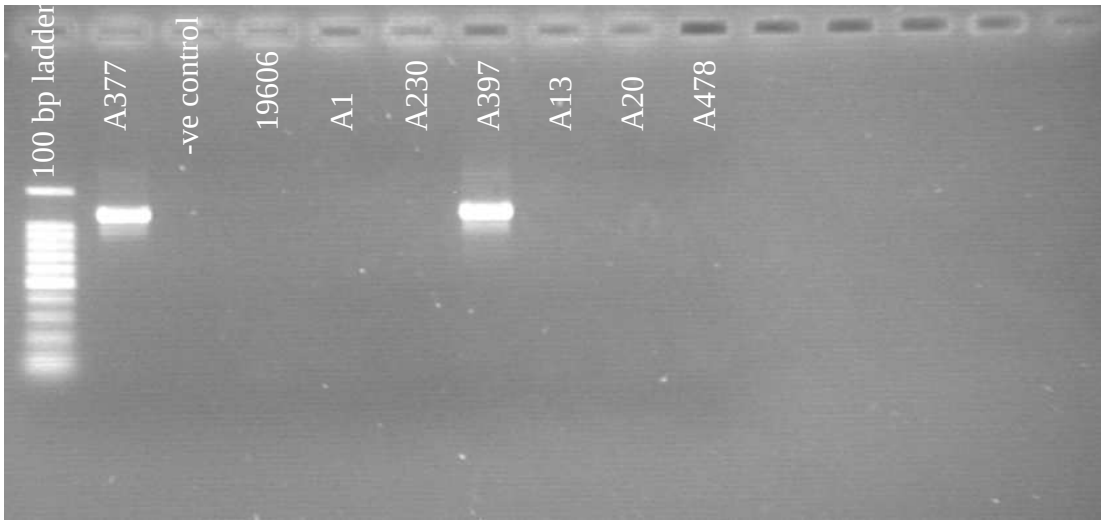

Figure 7: Example gel for the IS*Aba2* PCR. Lanes are labelled with markers, controls or isolate names.

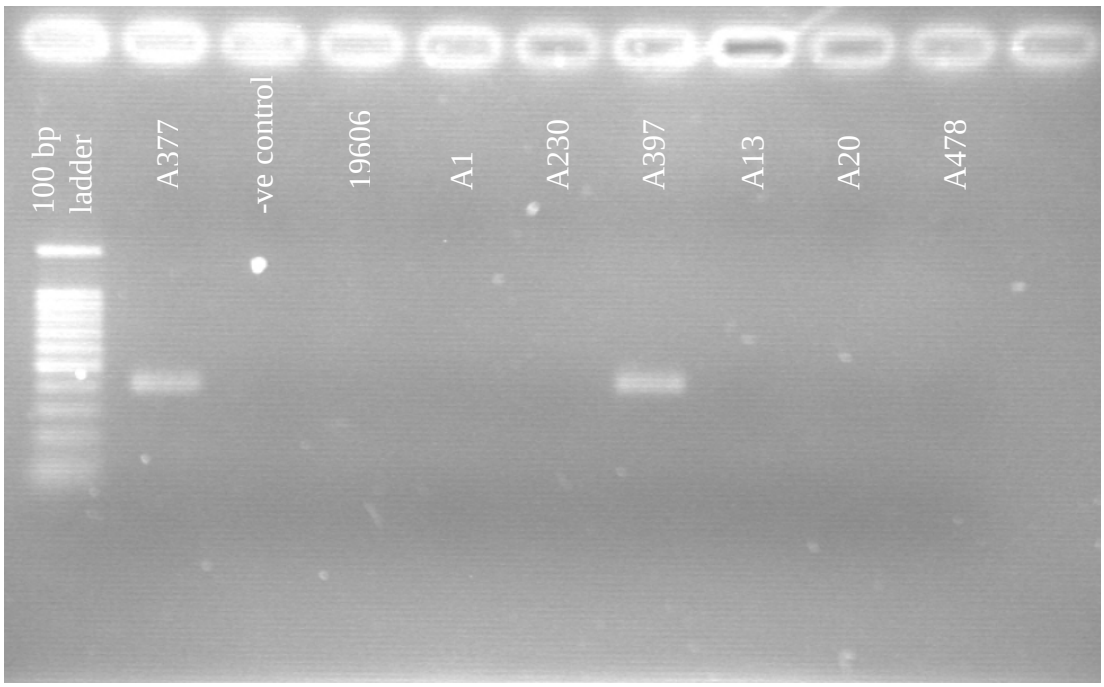

Figure 8: Example gel for the IS*Aba3* PCR. Lanes are labelled with markers, controls or isolate names.

3.1.2 Sequence group 2 isolates.

The isolates assigned to sequence group 2 (SG2) are shown in table 8. As with the SG1 isolates, there was no particular geographical grouping, with isolates identified from across Europe, Asia and South America. Of the 18 isolates in SG2, 12 carried the *bla*$_{\text{OXA-69}}$ gene (67%). The remaining six isolates possess one of three genes that have a maximum of one nucleotide difference (translating to one amino-acid difference) to *bla*$_{\text{OXA-69}}$. These three genes, *bla*$_{\text{OXA-107}}$, *bla*$_{\text{OXA-110}}$ and *bla*$_{\text{OXA-112}}$, were novel discoveries (see appendix A for sequence alignments). Two isolates carry a *bla*$_{\text{OXA-23-like}}$ gene, none carry a *bla*$_{\text{OXA-40-like}}$ gene, and one isolate carries a *bla*$_{\text{OXA-58-like}}$ gene. The three isolates encoding acquired carbapenemases are intermediately resistant or resistant to the carbapenems. IS*Aba1* was detected in 13 isolates (72%), though upstream of the *bla*$_{\text{OXA-51-like}}$ gene in only five. As with SG1 isolates, IS*Aba1*

Isolate	Origin	SG	*bla* $_{\text{OXA-51-like}}$ allele	IS*Aba1* upstream of *bla* $_{\text{OXA-51}}$	*bla* $_{\text{OXA-23-like}}$ allele	*bla* $_{\text{OXA-40-like}}$ allele	*bla* $_{\text{OXA-58-like}}$ allele	IS*Aba1*	IS*Aba2*	IS*Aba3*	MICs (mg/L)	
											IMP	MER
A424	Croatia	2	OXA-107	+	-	-	-	+	-	-	8	16
A479	UK import from Pakistan	2	OXA-69	-	+	-	-	+	-	-	8	8
A384	Oslo, Norway	2	OXA-69	-	-	-	+	+	-	+	4	2
A52	Freiburg, Germany	2	OXA-69	-	-	-	-	-	-	+	4	8
A472	Warsaw, Poland	2	OXA-107	+	-	-	-	+	-	-	4	8
A390	Pleven, Bulgaria	2	OXA-69	-	+	-	-	+	+	-	4	16
A404	Wroclaw, Poland	2	OXA-110	+	-	-	-	+	-	-	2	4
A443	Ljubljana, Slovenia	2	OXA-107	+	-	-	-	+	-	-	2	8
A442	Sofia, Bulgaria	2	OXA-69	-	-	-	-	+	-	-	1	2
A407	Wroclaw, Poland	2	OXA-110	+	-	-	-	+	-	-	1	8
A167	Bosnia	2	OXA-69	-	-	-	-	+	-	-	0.5	1
A15	Dordrecht, The Netherlands	2	OXA-69	-	-	-	-	+	-	-	0.5	0.5
A1	Nottingham, United Kingdom	2	OXA-69	-	-	-	-	-	-	-	0.25	0.5
A297	RUH 875 European clone I	2	OXA-69	-	-	-	-	+	-	-	0.25	0.5
A14	Venlo, The Netherlands	2	OXA-69	-	-	-	-	-	-	-	0.25	2
A63	Buenos Aires, Argentina	2	OXA-69	-	-	-	-	-	-	-	0.12	0.5
A335	Lincoln, United Kingdom	2	OXA-69	-	-	-	-	-	-	-	0.12	0.5
A368	Mansfield, United Kingdom	2	OXA-112	-	-	-	-	+	-	-	0.12	1
A329	Barcelona, Spain	3	OXA-71	-	-	+	-	-	-	-	64	>128
A94	Barcelona, Spain	3	OXA-71	-	-	-	-	-	-	-	8	4
A377	Regensburg, Germany	3	OXA-71	-	-	-	+	-	+	+	4	2
A13	Rotterdam, The Netherlands	3	OXA-71	-	-	-	-	-	-	-	1	2
A20	Paris, France	3	OXA-71	-	-	-	-	-	-	-	0.25	1
A478	LUH 5875 European clone III	3	OXA-71	-	-	-	-	-	-	-	0.25	2

Table 8: SG2 and SG3 isolates. IMP, imipenem; MER, meropenem; SG, sequence group.

was only detected upstream of the genes similar to *bla*$_{OXA-69}$ rather than *bla*$_{OXA-69}$ itself. One isolate contains IS*Aba2*, and this is one of the two isolates encoding a *bla*$_{OXA-23-like}$ gene, and two isolates contain IS*Aba3*, one of which is the isolate positive for *bla*$_{OXA-58}$.

3.1.3 Sequence group 3 isolates.

The isolates assigned to sequence group 3 (SG3) are listed in table 8. All of the SG3 isolates were obtained from Europe, though the sample size of six is quite small. All isolates in this group possess a *bla*$_{OXA-71}$ gene. None of the isolates possess a *bla*$_{OXA-23-like}$ gene. One isolate, with the highest MICs of imipenem and meropenem, carries a *bla*$_{OXA-40-like}$ gene. One isolate carries a *bla*$_{OXA-58-like}$ gene, and this is also the only isolate in which IS*Aba2* and IS*Aba3* were detected. IS*Aba1* was not detected in any isolate.

3.1.4 Isolates belonging to other sequence groups.

A total of 12 isolates belonged to less well represented sequence groups, and are shown in table 9. Three isolates belonged to sequence group 4 (SG4) as defined by Towner *et al* (2008) and possessed the *bla*$_{OXA-51}$ gene. IS*Aba1* was detected in all three isolates and was upstream of the *bla*$_{OXA-51}$ gene. Isolate A483 was also found to be positive for a *bla*$_{OXA-58-like}$ gene, IS*Aba2* and IS*Aba3*, and had a high imipenem MIC and intermediate meropenem MIC. Isolate A374 carried a *bla*$_{OXA-23-like}$ gene and IS*Aba2* and was carbapenem resistant.

Isolate	Origin	SG	*bla* OXA-51-like allele	IS*Aba1* upstream of *bla* OXA-51	*bla* OXA-23-like allele	*bla* OXA-40-like allele	*bla* OXA-58-like allele	IS*Aba1*	IS*Aba2*	IS*Aba3*	MICs (mg/L)	
											IMP	MER
A483	UK import from Morocco	4	OXA-51	+	-	-	+	+	+	+	128	4
A374	Rotterdam, The Netherlands	4	OXA-51	+	+	-	-	+	+	-	16	32
A125	Belfast, Northern Ireland	4	OXA-51	+	-	-	-	+	-	-	2	4
A376	Feldkirch, Austria	5	OXA-68	-	-	+	-	-	-	-	0.5	0.5
A388	Ioannina, Greece	6	OXA-92	-	-	-	+	+	+	+	1	2
A457	Estonia	7	OXA-106	-	-	-	-	-	-	-	0.5	1
A95	Trieste, Italy	10	OXA-98	-	-	-	-	-	-	-	0.12	0.25
A37	Singapore	8	OXA-64	-	-	-	-	+	-	-	0.25	0.25
A135	Ghent, Belgium	8	OXA-111	-	-	-	-	-	-	-	0.12	0.25
A92	Barcelona, Spain	9	OXA-69*	-	-	-	-	-	-	-	0.25	2
A187	Johannesburg, South Africa	9	OXA-68	-	-	-	-	+	-	-	0.12	1
A16	Leeds, United Kingdom	11	OXA-64	-	-	-	-	-	-	-	0.25	0.25

Table 9: Isolates assigned to sequence groups 4 to 11. IMP, imipenem; MER, meropenem; SG, sequence group. *Variant *bla*$_{\text{OXA-69}}$ sequence containing five silent nucleotide substitutions.

There was one isolate representing each of sequence groups 5, 6 and 7, as defined by Towner *et al* (2008). The SG5 isolate carried *bla*$_{\text{OXA-68}}$, and was positive only for a *bla*$_{\text{OXA-40-like}}$ gene, and no insertion sequences. The SG6 isolate contained a *bla*$_{\text{OXA-92}}$ gene. It was also found to carry a *bla*$_{\text{OXA-58-like}}$ sequence as well as IS*Aba1*, IS*Aba2* and IS*Aba3*, though IS*Aba1* was not upstream of the *bla*$_{\text{OXA-92}}$ gene. The isolate belonging to SG7 possessed *bla*$_{\text{OXA-106}}$, and was negative for all other acquired OXA-type carbapenemases and insertion sequences.

Four novel sequence groups were identified and named SG8, SG9, SG10 and SG11. The combination of amplicons defining these SGs is listed in table 10. Two representatives of SG8 were identified and they possess a *bla*$_{\text{OXA-64}}$ gene and a *bla*$_{\text{OXA-111}}$ gene. The *bla*$_{\text{OXA-111}}$ gene was a novel discovery. Isolate A37 was positive for IS*Aba1*, but otherwise both isolates were negative for all other OXA-type genes and insertion sequences. The two isolates belonging to SG9 were found to contain a *bla*$_{\text{OXA-69}}$ gene and a *bla*$_{\text{OXA-68}}$ gene. This was the only *bla*$_{\text{OXA-69}}$ gene that was not associated with an isolate belonging to SG2, however it contained five silent nucleotide substitutions ($G_{426}\rightarrow A$, $C_{474}\rightarrow A$, $C_{511}\rightarrow T$, $G_{540}\rightarrow A$ and $T_{801}\rightarrow C$) compared to the *bla*$_{\text{OXA-69}}$ gene found in the SG2 isolates. A *bla*$_{\text{OXA-68}}$ gene was also found in the single SG5 isolate in this study. As SG5 and SG9 only differ by the presence of a band for the *bla*$_{\text{OXA-51-like}}$ gene in the group 2 multiplex PCR, it is possible that this isolate is, in fact, another member of SG5. The *bla*$_{\text{OXA-68}}$-containing isolate was positive for IS*Aba1*, otherwise both isolates were negative for all other products. One isolate was found for each of SG10 and SG11. The SG10 isolate carries a *bla*$_{\text{OXA-98}}$ gene, while the SG11 isolate carries a *bla*$_{\text{OXA-64}}$ gene. A *bla*$_{\text{OXA-64}}$

	Group 1 Multiplex PCR			Group 2 Multiplex PCR		
SG	*csuE*	*bla* $_{\text{OXA-51-like}}$	*ompA*	*csuE*	*bla* $_{\text{OXA-51-like}}$	*ompA*
1	+	+	+	-	-	-
2	-	-	-	+	+	+
3	+	+	-	-	-	+
4	-	+	+	-	-	-
5	-	-	+	-	-	-
6	+	-	-	-	+	+
7	-	-	+	+	-	-
8	+	+	-	-	-	-
9	-	-	+	-	+	-
10	+	-	+	-	-	-
11	-	+	-	-	-	-

Table 10: Sequence group designation by amplicons. SG, sequence group; *csuE*, gene encoding a product important in a pilus chaperone-usher secretion system; *ompA*, gene encoding the porin outer-membrane protein A. SG1, 2 and 3 definitions by Turton *et al* (2007), and SG4, 5, 6 and 7 definitions by Towner *et al* (2008).

gene was also found in an isolate belonging to SG8, however SG8 and SG11 only differ in the amplification of a *csuE* product in the group 1 multiplex PCR, so are not that distantly related. No other OXA-type genes or insertion sequences were found in either isolate.

It should be noted that while all isolates carried a *bla*$_{\text{OXA-51-like}}$ gene, they did not all give amplicons using the *bla*$_{\text{OXA-51-like}}$ primers used in the sequence grouping scheme. This is because the primers were designed to be specific for particular *bla*$_{\text{OXA-51-like}}$ subgroups. For example, the group 1 multiplex PCR primers for the *bla*$_{\text{OXA-51-like}}$ genes contain a total of four mismatches when compared to the sequence for *bla*$_{\text{OXA-69}}$, whereas they match completely with *bla*$_{\text{OXA-66}}$.

3.2 Pulsed-field gel electrophoresis compared with sequence grouping and *bla*$_{\text{OXA-51-like}}$ gene content.

The 64 isolates in this study were analysed by PFGE, the majority of which was performed by Dr. Hamouda. PFGE profile analysis revealed that the isolates varied considerably, with very few distinct groups (figure 9). Six pairs of isolates and two groups of three isolates clustered at ≥87% similarity, which is a cut-off value that has been suggested for use in identifying isolates belonging to the same epidemic strain (Seifert *et al*., 2005). The first group of three isolates contains A24, A6 and A4, which were all isolated from Nottingham, UK, all possess a *bla*$_{\text{OXA-66}}$ gene and belong to SG1. Similarly, the other group of three closely related isolates is composed of A13, A20 and A478, which all belong to SG3 and possess a *bla*$_{\text{OXA-71}}$ gene. However, their geographical distribution is more widespread, though still limited to mainland Europe. Three of the six closely-related pairs of isolates also carry the same *bla*$_{\text{OXA-51-like}}$ gene as one another and belong to the same SG. However, the other three pairs, while belonging to the same sequence group, possess different *bla*$_{\text{OXA-51-like}}$ genes. A480, A369 and A186 all carry *bla*$_{\text{OXA-66}}$, while their closely related isolates A484, A401 and A343 carry *bla*$_{\text{OXA-83}}$, *bla*$_{\text{OXA-82}}$ and *bla*$_{\text{OXA-109}}$ respectively. However, these genes only differ from *bla*$_{\text{OXA-66}}$ by one nucleotide (*bla*$_{\text{OXA-83}}$ and *bla*$_{\text{OXA-109}}$), or two nucleotides with one being a silent substitution (*bla*$_{\text{OXA-82}}$), in all cases translating to one amino-acid difference in the enzyme (see appendix A).

The majority of isolates (53%) shared ≤80% similarity. Interestingly some isolates with different *bla*$_{\text{OXA-51-like}}$ sequences, and belonging to different sequence groups,

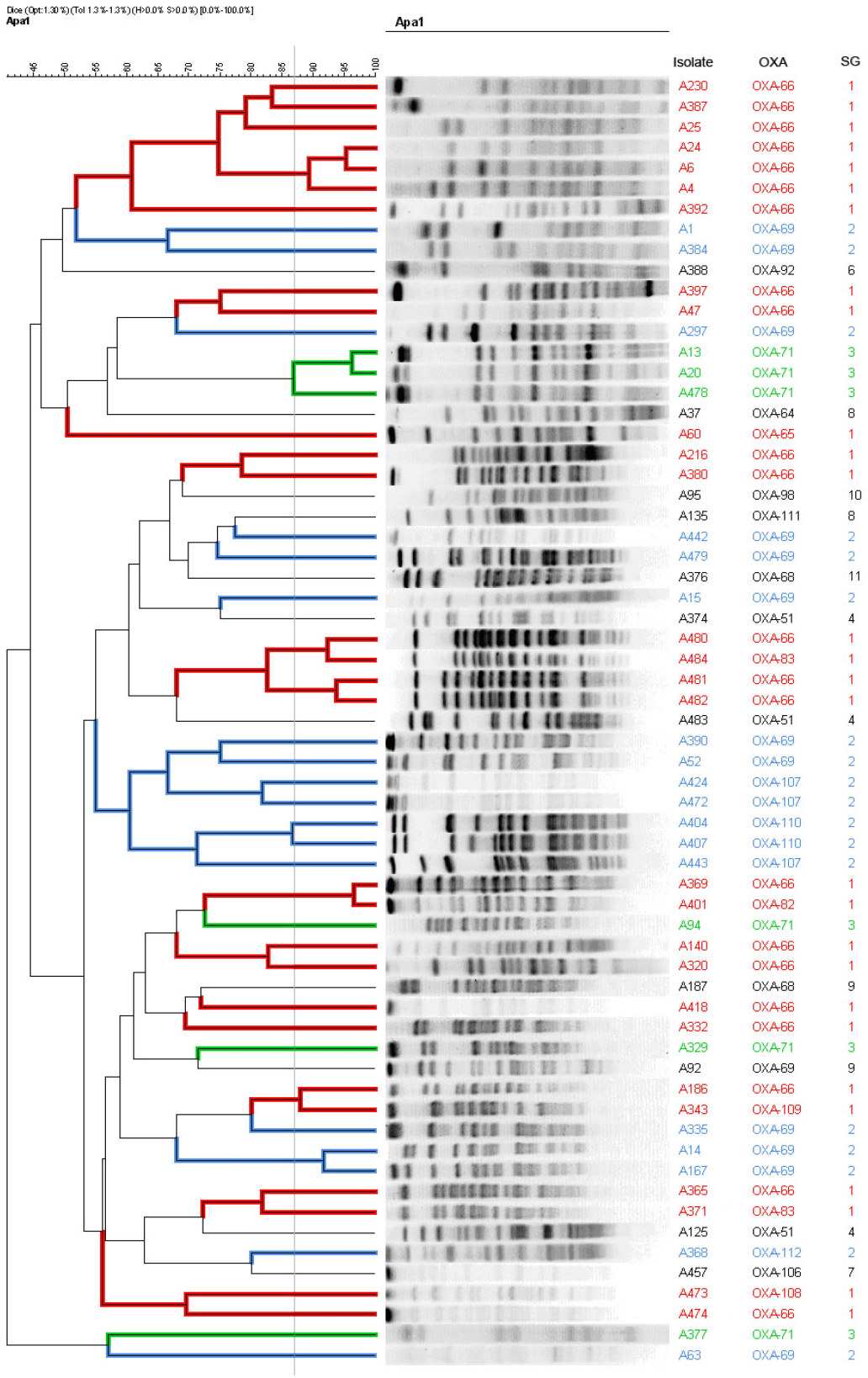

Figure 9: Pulsed-field gel electrophoresis analysis. OXA, *bla*$_{\text{OXA-51-like}}$ gene; SG, sequence group. A grey vertical line denotes relatedness of 87%. SG1 isolates are in red, SG2 isolates in blue and SG3 isolates in green.

were more similar to one another according to PFGE analysis than they were to isolates containing the same *bla*$_{\text{OXA-51-like}}$ gene and belonging to the same sequence group. Thus, isolate A186, which belonged to SG1 and contained a *bla*$_{\text{OXA-66}}$ gene, shared 80% identity with isolate A335, which belonged to SG2 and contained a *bla*$_{\text{OXA-69}}$ gene. Similarly, isolate A368, which belonged to SG2 and contained a *bla*$_{\text{OXA-112}}$ gene, shared 80% identity with isolate A457, which contained a *bla*$_{\text{OXA-106}}$ gene and belonged to SG7. Conversely, isolates with the same *bla*$_{\text{OXA-51-like}}$ gene and SG were not all grouped together: for example, of the five isolates belonging to SG3 encoding *bla*$_{\text{OXA-71}}$, three are clustered together and related at 87%. The other two isolates are clustered nearest to some SG1 isolates in the case of A94 or a SG2 isolate in the case of A329, are about 61% related to one another and <45% related to the other three SG3 isolates. When isolates were from the same location, contain the same *bla*$_{\text{OXA-51-like}}$ gene, and belong to the same sequence group, they tend to form more closely related clusters. This can be seen with isolates A480, A484, A481 and A482 from Nottingham, UK. These isolates represent a dominant lineage in the particular hospital from which the isolates were taken. In general, the mismatch between the PFGE profile on the one hand, and the *bla*$_{\text{OXA-51-like}}$ gene content and SG designation on the other, was considerable.

3.3 Carbapenem susceptibility testing.

Out of the 64 isolates included in the study, 36 (56%) were susceptible to both imipenem and meropenem according to BSAC criteria (MIC ≤2 mg L^{-1}). Resistance to imipenem and meropenem (MIC ≥16 mg L^{-1}) was seen in six isolates, four isolates were resistant to meropenem and intermediate (MIC of 4 to 8 mg L^{-1}) for imipenem,

one isolate was resistant to imipenem and intermediate to meropenem, nine isolates were intermediate for imipenem and meropenem, three isolates were intermediate for imipenem only, and five isolates were intermediate for meropenem only. Of the six imipenem- and meropenem-resistant isolates, four belonged to SG1, one to SG3 and one to SG4. These six isolates possessed a *bla*$_{\text{OXA-23-like}}$ gene (one isolate), a *bla*$_{\text{OXA-40-like}}$ gene (three isolates), a *bla*$_{\text{OXA-58-like}}$ gene (one isolate) or carried IS*Aba1* upstream of the *bla*$_{\text{OXA-51-like}}$ gene (two isolates). The four isolates that were resistant to meropenem with intermediate susceptibilities to imipenem either had IS*Aba1* upstream of the *bla*$_{\text{OXA-51-like}}$ gene (3 isolates) or carried a *bla*$_{\text{OXA-23-like}}$ gene (1 isolate). The single isolate that was resistant to imipenem with intermediate resistance to meropenem possessed a *bla*$_{\text{OXA-58-like}}$ gene and IS*Aba1* upstream of the *bla*$_{\text{OXA-51-like}}$ gene. Of the 28 isolates that were resistant or of intermediate resistance to one or both carbapenems, 13 belonged to SG1 (46%), 9 belonged to SG2 (32%), 3 were from SG3 (11%), and SG4 accounted for the remaining 3 isolates (11%). Within the 36 isolates that were fully susceptible, only one carried an acquired OXA-type β-lactamase (a *bla*$_{\text{OXA-58-like}}$ gene in A388), and none were positive for IS*Aba1* upstream of the *bla*$_{\text{OXA-51-like}}$ gene. All isolates had previously been screened by Dr. Towner for the presence of *bla*$_{\text{IMP-like}}$ and *bla*$_{\text{VIM-like}}$ metallo-β-lactamases, with only isolate A474 being positive, and was found to carry *bla*$_{\text{VIM-2}}$.

A total of 13 isolates possess an acquired OXA-type carbapenemase gene of either the *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-40-like}}$ or *bla*$_{\text{OXA-58-like}}$ families that did not also possess an IS*Aba1* sequence upstream of their *bla*$_{\text{OXA-51-like}}$ gene. There are a total of 11 isolates for which the opposite is the case. In order to determine whether isolates encoding a

gene for an acquired OXA-type carbapenemase are associated with higher carbapenem MICs than isolates that contain an IS*Aba1* element upstream of their *bla*$_{\text{OXA-51-like}}$ gene, a Mann-Whitney U test was conducted on both the imipenem and meropenem MICs for these isolates, and the results are shown in table 11. The *p* values for both imipenem and meropenem are greater than 0.05, demonstrating that there is no statistical difference in the MICs of imipenem and meropenem for isolates with an acquired OXA-type carbapenemase to those with IS*Aba1* upstream of their *bla*$_{\text{OXA-51-like}}$ gene.

		Number	Mean rank	Sum of Ranks	Probability[a]	Probability[b]
Imipenem MICs	Acquired OXA	13	13.73	178.5	0.361	0.345
	IS*Aba1* -OXA-51	11	11.05	121.5		
	Total	24				
Meropenem MICs	Acquired OXA	13	11.92	155	0.691	0.659
	IS*Aba1* -OXA-51	11	13.18	145		
	Total	24				

Table 11: Summary of results for Mann-Whitney U test for the difference between the imipenem and meropenem MICs for isolates with an acquired OXA-type carbapenemase without an IS*Aba1* upstream of their *bla*$_{\text{OXA-51-like}}$ gene, and vice versa. Number, number of isolates; Mean rank, the mean rank value for the MICs for the isolates; Sum of Ranks, the sum of all of the ranks for the MICs for the isolates, [a]*p* value not corrected for ties; [b]*p* value corrected for ties.

3.4 OXA-51-like functional map.

The OXA-51-like enzyme map revealed the enzymes formed distinct groupings (figure 10). Three very closely inter-related enzyme groups, with each member separated by one or two amino-acid differences were formed around OXA-66, OXA-69 and OXA-98. These three enzymes appeared to form a hub from which the enzymes in their respective groups radiated. Outside of these major groups, four other enzymes had more than one enzyme that was closely related to them branching from them. Enzymes OXA-71, the enzyme representative of SG3, and OXA-51, the enzyme representative of SG4, both had two similar enzymes radiating from them. Enzymes OXA-106, found in the single representative of SG7, and OXA-90 both had three branches leading from them to similar enzymes. All other enzymes were linked in a chain-like manner rather than being clustered. The degree of variation within the OXA-51-like enzymes as a whole is considerable, with the two most distantly related enzymes, OXA-87 and OXA-75, differing by 16 amino-acids (for amino-acid sequences see appendix A).

The three enzymes representative of SG1 (OXA-66), SG2 (OXA-69) and SG3 (OXA-71) were not closely related, with differences at four amino-acid positions between OXA-66 and OXA-69, five amino-acid positions between OXA-66 and OXA-71, and seven amino-acid positions between OXA-69 and OXA-71. The OXA-66 cluster was the largest group with 14 members (OXA-66, -65, -88, -76, -109, -82, -83, -84, -79, -80, -115, -131, -138 and –EU255296). The OXA-69 cluster had five members (OXA-69, -92, -107, -110 and -112), and 11 enzymes comprised the OXA-98 cluster (OXA-98, -91, -68, -77, -78, -116, -117, -128, -144, -149 and -150). In this

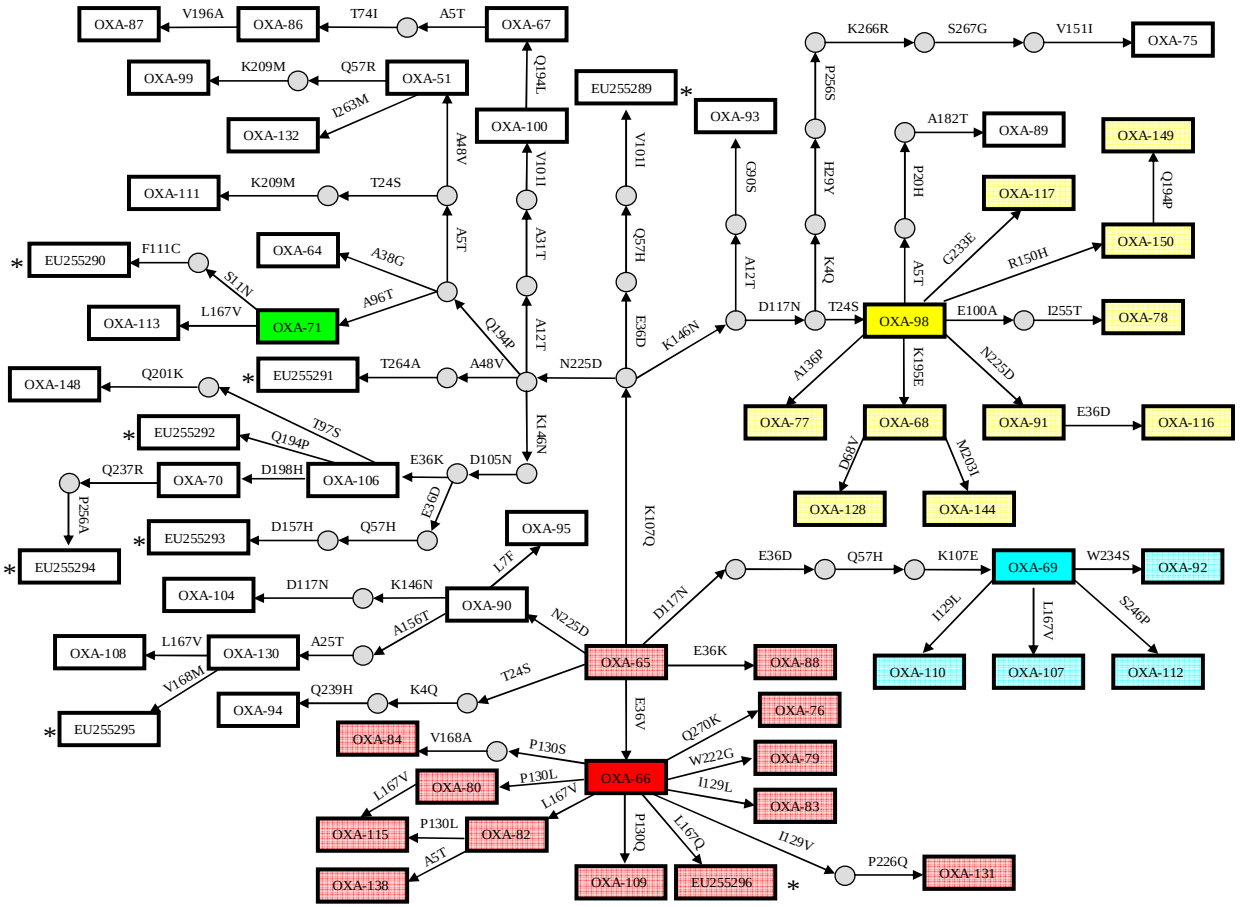

Figure 5: OXA-51-like enzyme map. Amino-acid substitutions are listed with respect to OXA-65. *PubMed accession number is listed for these enzymes as their official designation has yet to be publicly released. Grey circles represent hypothetical intermediate enzymes. Solid red, OXA-66; pale red, enzymes related to OXA-66; solid blue, OXA-69; pale blue, enzymes related to OXA-69; solid yellow, OXA-98; pale yellow, enzymes related to OXA-98; green, OXA-71.

study, all isolates that were identified as belonging to SG1 carried genes for OXA-66-like enzymes, except for isolate A473, which carries *bla*$_{\text{OXA-108}}$. All isolates from SG2 had genes for OXA-69-like enzymes, and the gene for OXA-71 was identified in all SG3 isolates.

Interestingly within different enzyme groups, the same amino-acid variations were observed. Changes at position 167 from a leucine residue (with respect to OXA-65) to either a valine or a glutamine residue is seen three times within the OXA-66-like cluster, once in the OXA-69-like cluster, once between OXA-71 and OXA-113, and once between OXA-130 and OXA-108. Similarly, differences at position 129 between an isoleucine and a leucine or valine are seen twice in the OXA-66-like cluster and once in the OXA-69-like cluster. The enzyme OXA-65 was chosen as the reference enzyme, which all amino-acid differences would be listed with respect to, as it appears to be the most central enzyme within the map. The central area of the map is bereft of enzymes, and it is possible that the ancestor of the OXA-51-like enzyme family would have taken up a position in this region. It is also of note that the enzymes that have been discovered more recently, identifiable by having the higher numerical designations or just the accession numbers, tend to be located on the fringes of the map rather than more centrally, indicating that in some cases we may be observing the evolution of these enzymes in real time.

3.5 Multi-locus sequence typing.

3.5.1 Analysis of concatenated sequences for all seven loci.

A total of 44 isolates were chosen for analysis by MLST. Isolates were chosen such that at least one isolate with every different *bla*$_{\text{OXA-51-like}}$ gene identified in this study was included, and the more prevalent SGs were proportionally represented. The designations of the alleles for the seven genes amplified for the 44 isolates along with their sequence type (ST) designation are shown in table 12. Provisional ST designations were assigned for novel allele combinations that were not found on the online *A. baumannii* MLST database.

A total of 24 different STs were identified within the 44 isolates, with 19 of these being novel. Analysis of the data using the eBURST method, designed for examining evolutionary descent using MLST data (Feil *et al*, 2004), identified four clonal complexes along with nine singletons (figure 11). The largest clonal complex contained ST34 which was identified as a potential founder, with ST46, ST36, ST40, ST4 and ST22 radiating from it. A second major clonal complex contained ST49, ST51, ST47 and ST25 surrounding ST33, which was identified as a potential founder for the complex. The other two minor clonal complexes identified contained ST50 and ST48, and ST35 and ST38 respectively.

The index of association standardised (I_A^S) was calculated to determine the linkage disequilibrium between the seven loci (Haubold *et al*, 1998). This measure calculates whether within a population, sets of alleles are linked together i.e. there is linkage disequilibrium, or if alleles are randomly distributed with respect to one another

Isolate	*gltA*	*gyrB*	*gdhB*	*recA*	*cpn60*	*gpi*	*rpoD*	ST
A92	2	2	2	1	1	2	7	2
A230	1	12	3	2	2	7	3	4
A397	1	12	3	2	2	7	3	4
A187	1	15	13	12	4	12	2	20
A483	1	15	13	12	4	12	2	20
A371	1	3	3	2	2	7	3	22
A401	1	3	3	2	2	7	3	22
A484	1	3	3	2	2	7	3	22
A384	10	12	4	11	1	9	5	25
A1	10	12	4	11	4	9	5	33*
A297	10	12	4	11	4	9	5	33*
A368	10	12	4	11	4	9	5	33*
A442	10	12	4	11	4	9	5	33*
A63	10	12	4	11	4	9	5	33*
A216	1	12	3	2	2	3	3	34*
A24	1	12	3	2	2	3	3	34*
A25	1	12	3	2	2	3	3	34*
A320	1	12	3	2	2	3	3	34*
A392	1	12	3	2	2	3	3	34*
A4	1	12	3	2	2	3	3	34*
A6	1	12	3	2	2	3	3	34*
A20	1	1	1	1	1	9	6	35*
A329	1	1	1	1	1	9	6	35*
A377	1	1	1	1	1	9	6	35*
A478	1	1	1	1	1	9	6	35*
A387	1	12	3	2	2	4	3	36*
A388	10	26	4	11	4	11	5	37*
A13	1	27	1	1	1	9	6	38*
A37	1	15	2	15	1	16	18	39*
A47	1	12	3	2	2	19	3	40*
A60	1	15	18	10	14	12	18	41*
A473	1	24	22	12	17	20	4	42*
A457	1	25	13	6	4	25	22	43*
A135	1	28	21	10	1	24	4	44*
A95	14	23	20	16	1	11	20	45*
A343	1	3	3	2	2	3	3	46*
A479	10	12	4	11	4	22	5	47*
A125	12	18	12	1	15	3	19	48*
A404	10	12	4	11	4	4	5	49*
A407	10	12	4	11	4	4	5	49*
A443	10	12	4	11	4	4	5	49*
A472	10	12	4	11	4	4	5	49*
A374	12	18	12	1	15	26	19	50*
A424	10	12	4	11	4	19	5	51*

Table 12: Multi-locus sequence typing alleles amplified for 44 isolates. ST, sequence type. * Provisional designation.

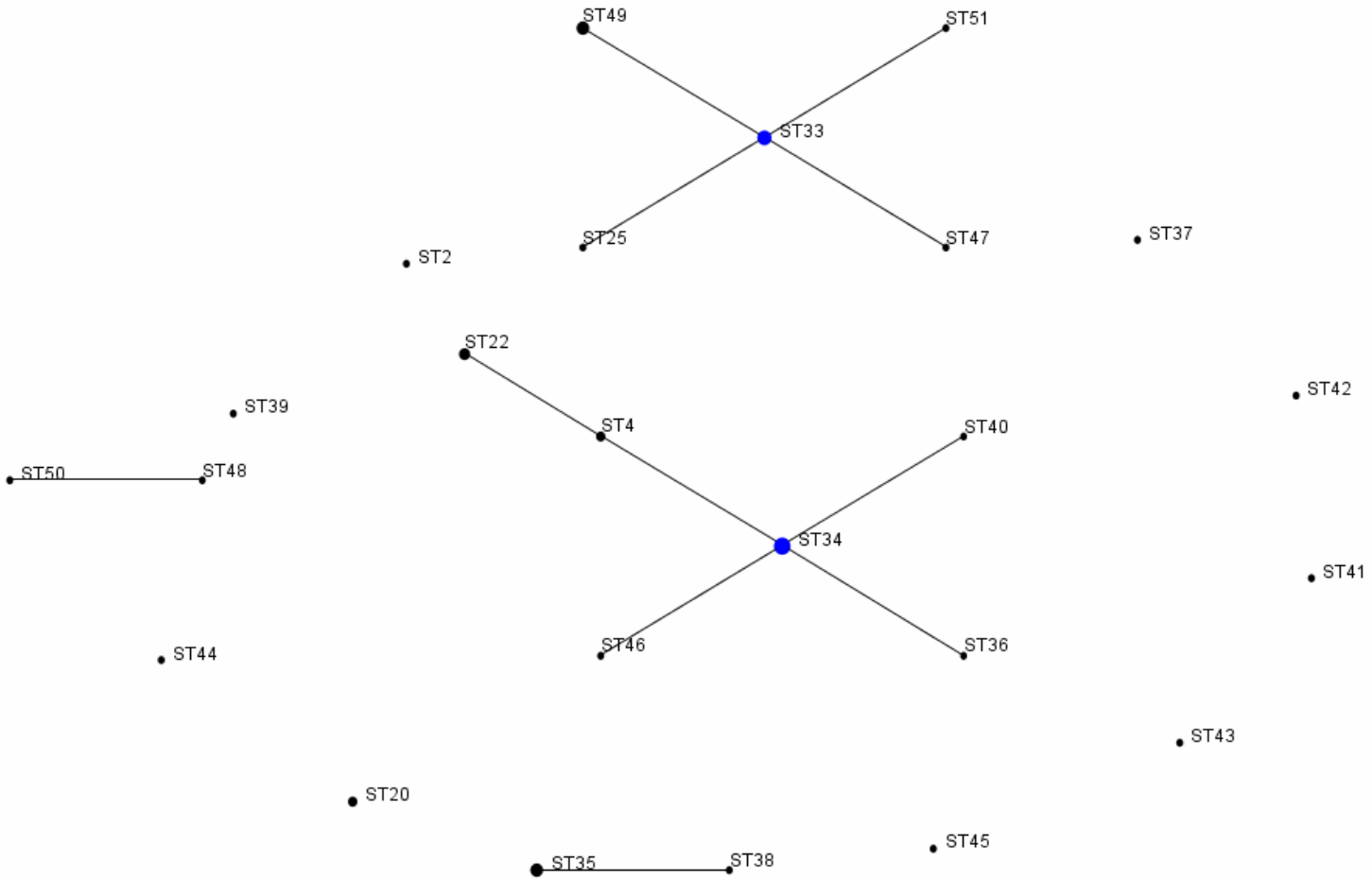

Figure 11: eBURST analysis of the 44 isolates. The size of the dot is proportional to the number of isolates within the sequence type. ST34 and ST33 are proposed clonal complex founders (blue).

throughout the population i.e. there is linkage equilibrium. Linkage disequilibrium arises through clonal spread in a population, while the presence of frequent recombination will result in linkage equilibrium. Complete linkage equilibrium will result in $I_A^S = 0$, while complete linkage disequilibrium will result in I_A^S significantly greater than 0. An I_A^S value that differs significantly from 0 is considered to represent linkage disequilibrium (Maynard Smith *et al*, 1993). Analysis of the entire dataset of 44 isolates produced an I_A^S value of 0.4907 ($p = <0.001$). This was decreased to 0.3569 ($p = <0.001$) when only one representative of each sequence type was included. Both values are significantly greater than 0, and indicate that the population is clonal.

A Neighbour-joining tree was constructed using concatenated sequences for the seven genes for all 44 isolates, and is shown in figure 12, along with the OXA-51-like enzyme carried by each isolate. The tree is split into two main clades. All of the isolates encoding genes for an OXA-66-like enzyme, and belonging to SG1, are grouped together in the top clade of the tree with reasonable support. Similarly, the five isolates encoding genes for OXA-71 from SG3 are grouped together in the bottom clade of the tree with a high degree of support. However, the isolates from SG2 that possess genes for OXA-69-like enzymes were split between the two clades. Eight of these isolates were grouped in the top clade with a good degree of support, while the other six isolates were found in the bottom clade, with a high level of support for their relatedness to the SG3 isolates. In order to determine the reason for the splitting of the SG2 isolates, neighbour-joining trees were constructed for each of the seven loci individually.

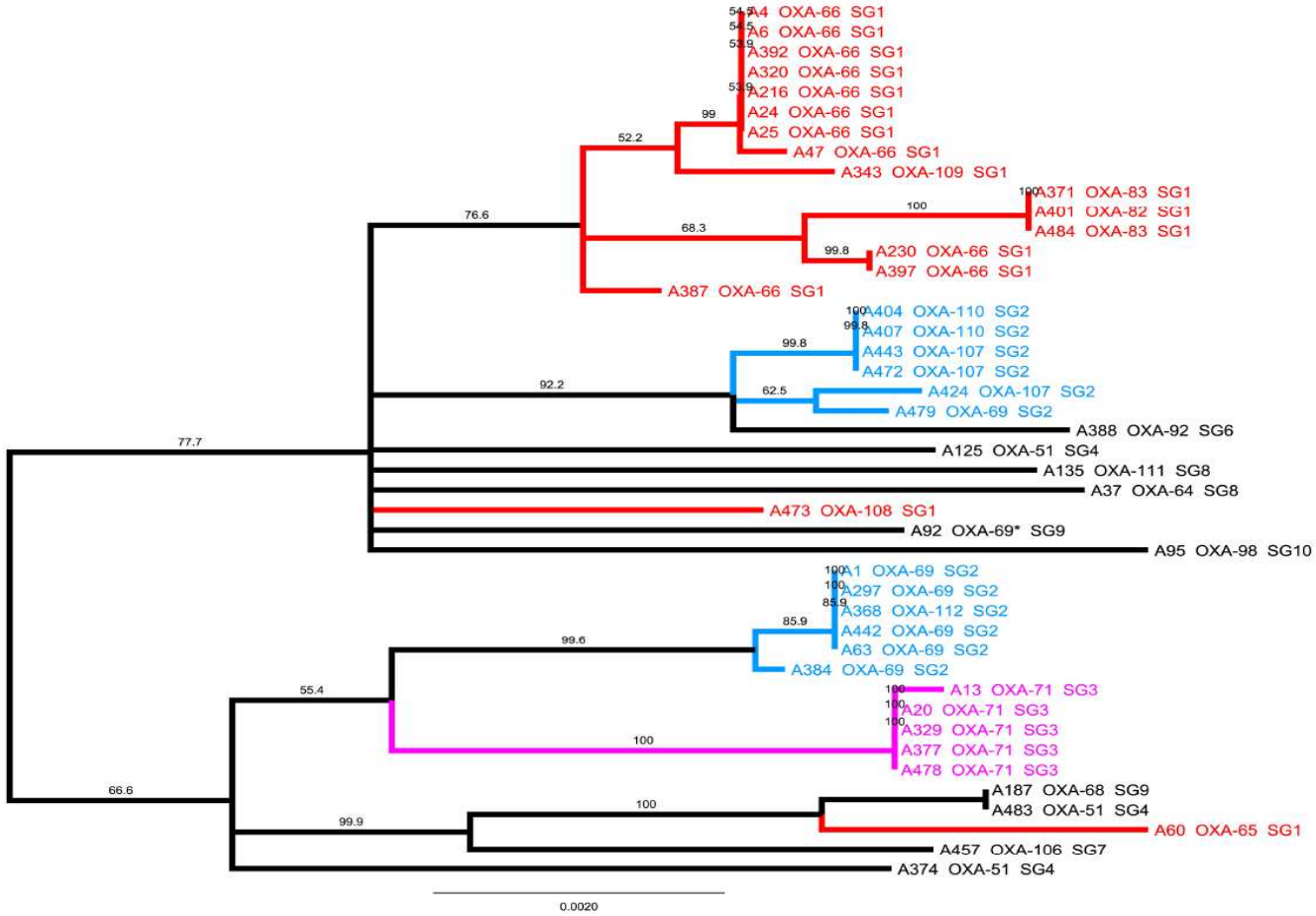

Figure 12: Neighbour-joining tree for 44 isolates using concatenated sequences for the seven genes. Isolate names are followed by the OXA-51-like enzyme that they carry and their SG. Branches are labelled with % support. The tree is mid-point rooted. Red, SG1; blue, SG2; pink, SG3.

3.5.2 Analysis of each individual locus.

The neighbour-joining trees for the seven MLST genes are shown in figures 13 to 19. Comparison of the tree topologies for all identified alleles of the seven genes revealed inconsistencies with the trees for *gyrB* and *gpi* in relation to one another and to the other five trees. Within the trees for the *cpn60*, *rpoD*, *gltA*, *recA* and *gdhB* alleles, isolates forming SG1, SG2 and SG3 generally cluster with the other isolates forming their SG. In some instances, for example in the *gdhB* tree (figure 16), the isolates from these three main SGs form their own discrete clades. In others, for example the *gltA* tree (figure 13), SG2 is in a distinct clade while SG1 and SG3 share one. Conversely, in the *rpoD* tree (figure 19) SG3 is found in its own clade, while SG1 and SG2 share one. While there is some variation within the phylogenies for these five genes, the data from these alleles do not result in a major splitting of members from the same sequence group across two clades. However, the phylogenies for the *gyrB* and *gpi* alleles are different. The tree showing the relationships between the *gpi* alleles (figure 14) shows a major split between isolates belonging to SG2. Isolates A424, A472, A443, A407, A404 and A479 are found in the top half of the tree, which they share with the SG1 isolates. However, isolates A63, A442, A384, A368, A297 and A1 are found in the lower half of the tree along with the SG3 isolates. The branches for both parts of the tree show strong support. This major split of the SG2 isolates did not match with the features observed for the first five genes mentioned above. Analysis of the tree for the *gyrB* alleles (figure 18) revealed further inconsistencies. The majority of the isolates belonging to SG1 are found in the top half of the tree close to the SG2 isolates. However, four of the SG1 isolates, A484, A401, A371 and A343, are in the lower region of the tree, with strong

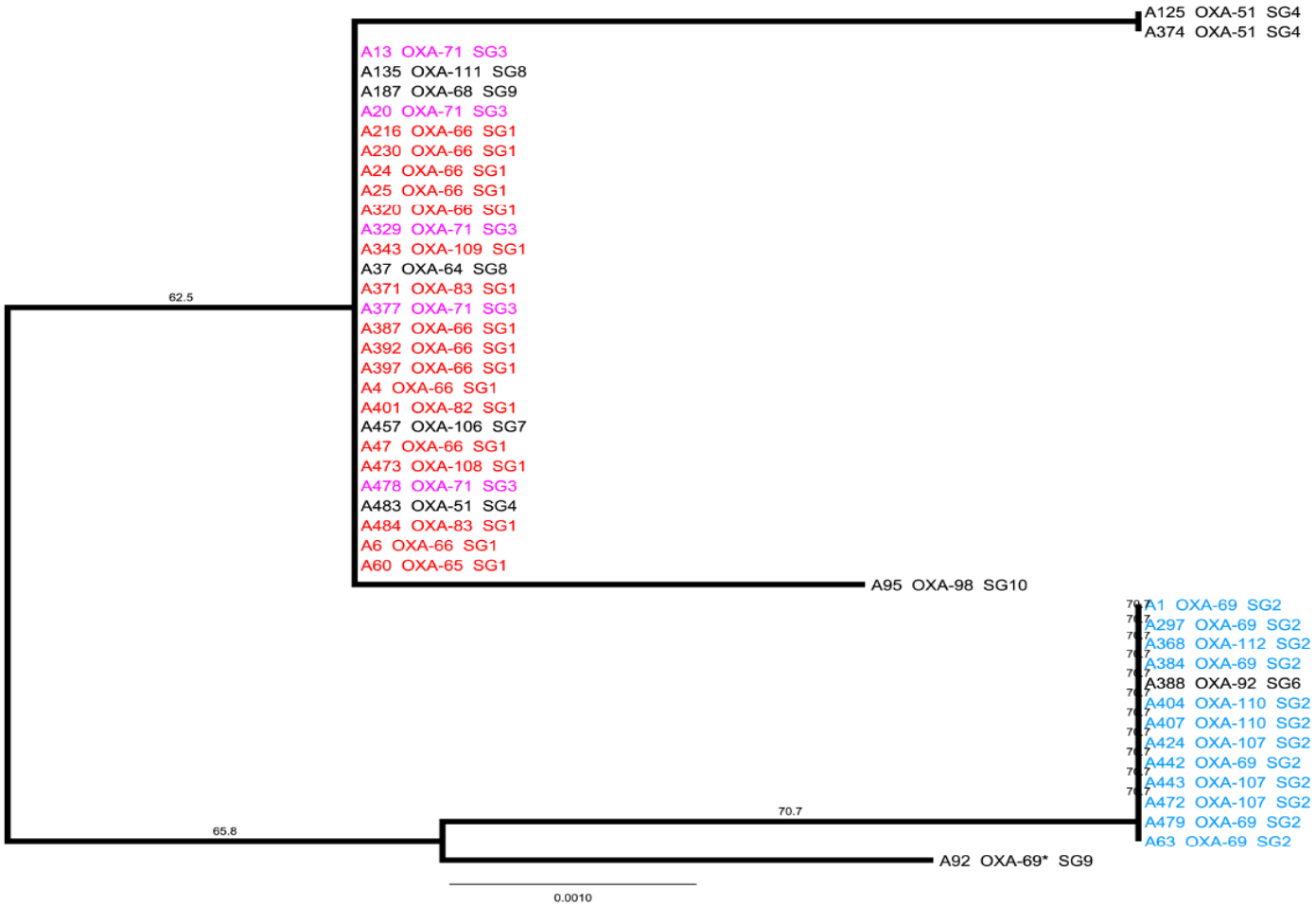

Figure 13: *gltA* neighbour-joining tree. Isolate names are followed by the OXA-51-like enzyme that they carry and their SG. Branches are labelled with % support. The tree is mid-point rooted. Red, SG1; blue, SG2; pink, SG3.

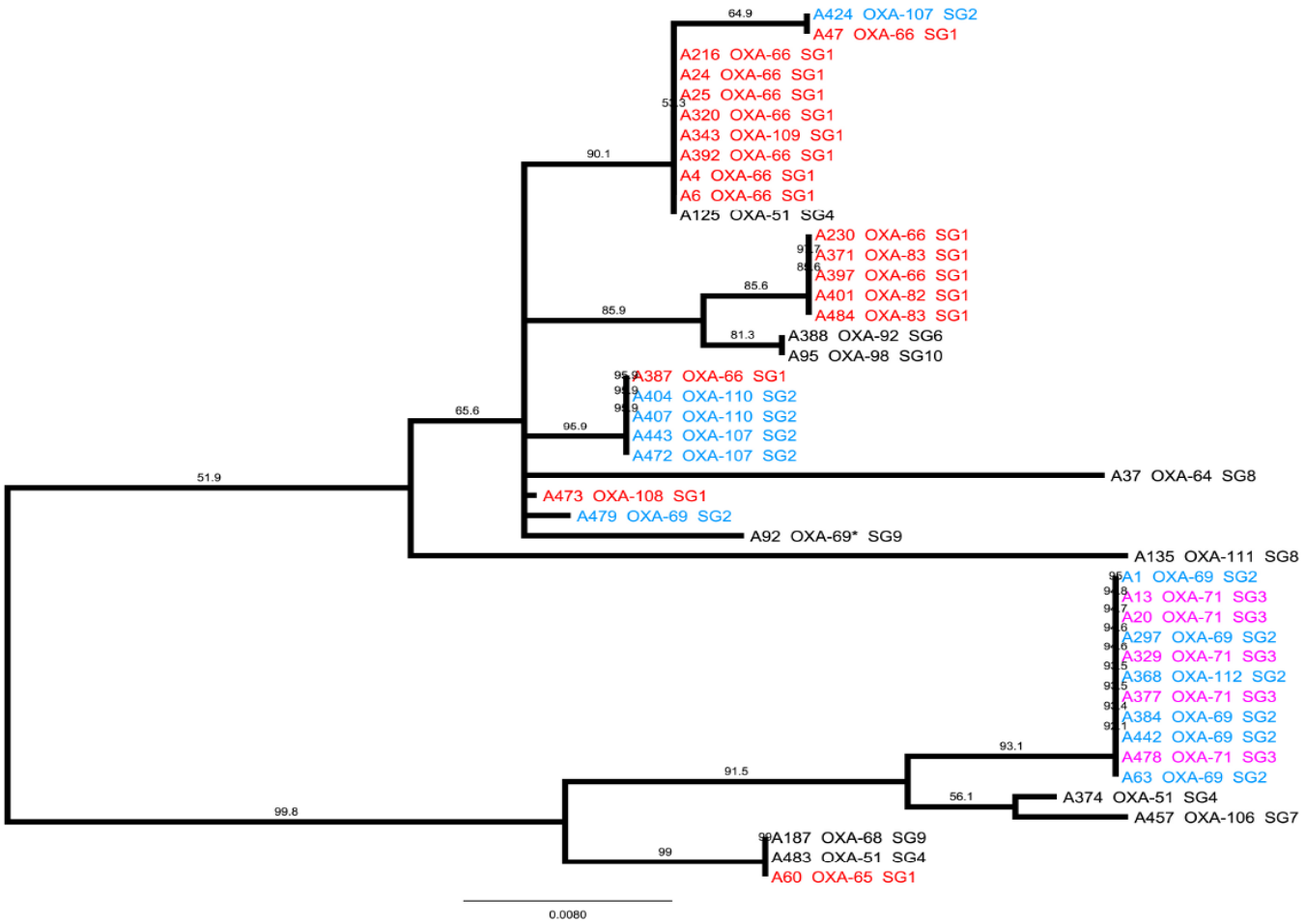

Figure 14: *gpi* neighbour-joining tree. Isolate names are followed by the OXA-51-like enzyme that they carry and their SG. Branches are labelled with % support. The tree is mid-point rooted. Red, SG1; blue, SG2; pink, SG3.

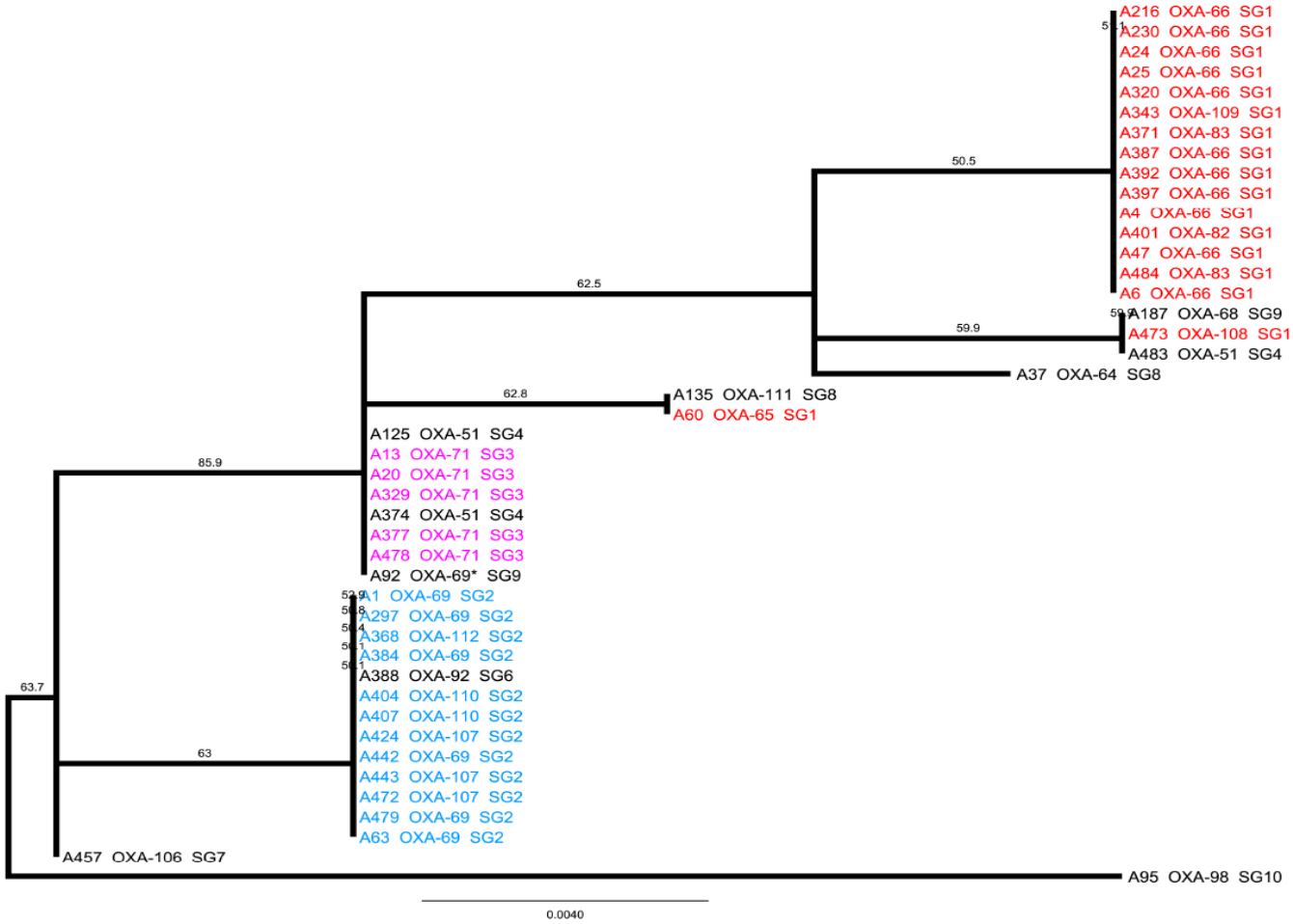

Figure 15: *recA* neighbour-joining tree. Isolate names are followed by the OXA-51-like enzyme that they carry and their SG. Branches are labelled with % support. The tree is mid-point rooted. Red, SG1; blue, SG2; pink, SG3.

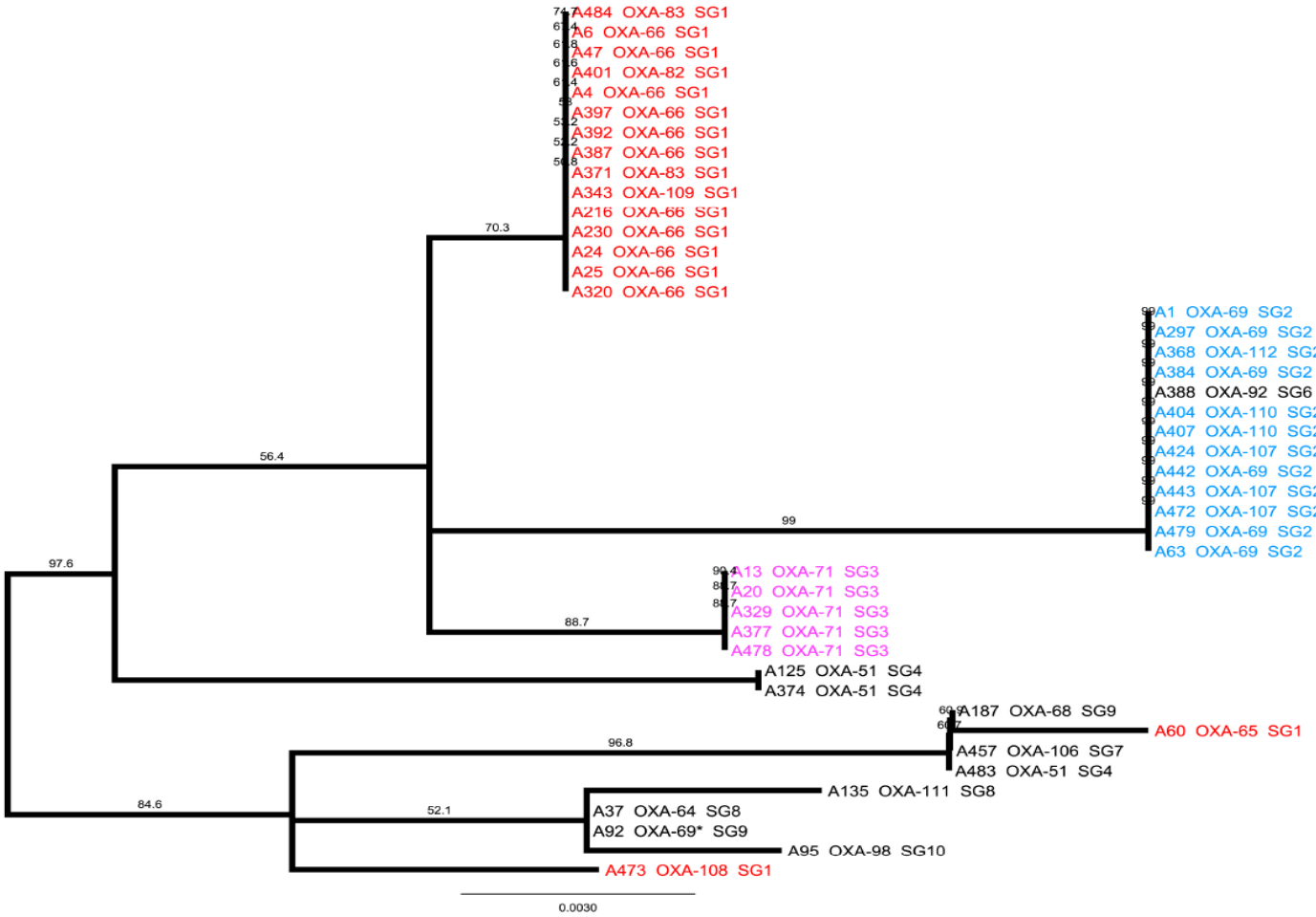

Figure 16: *gdhB* neighbour-joining tree. Isolate names are followed by the OXA-51-like enzyme that they carry and their SG. Branches are labelled with % support. The tree is mid-point rooted. Red, SG1; blue, SG2; pink, SG3.

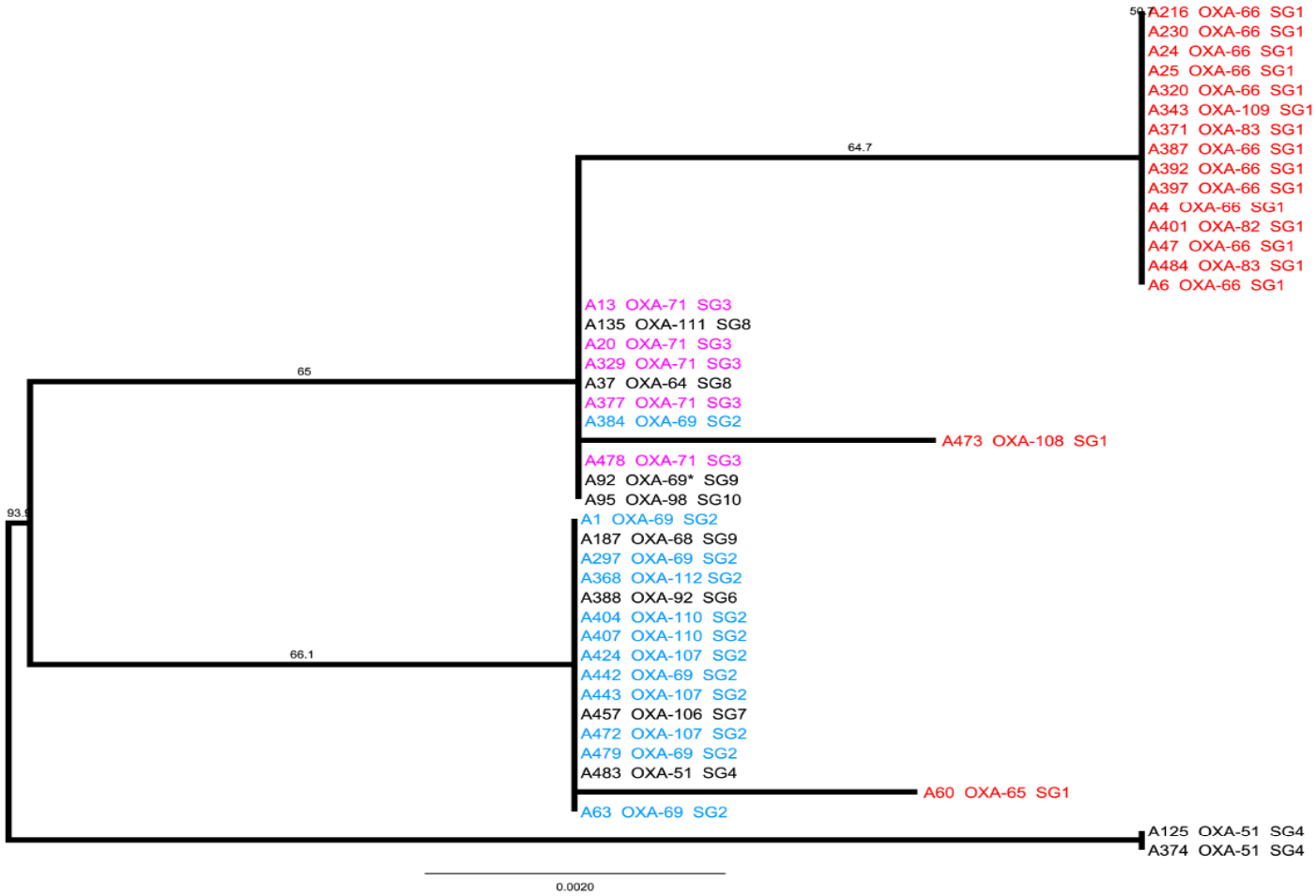

Figure 17: *cpn60* neighbour-joining tree. Isolate names are followed by the OXA-51-like enzyme that they carry and their SG. Branches are labelled with % support. The tree is mid-point rooted. Red, SG1; blue, SG2; pink, SG3.

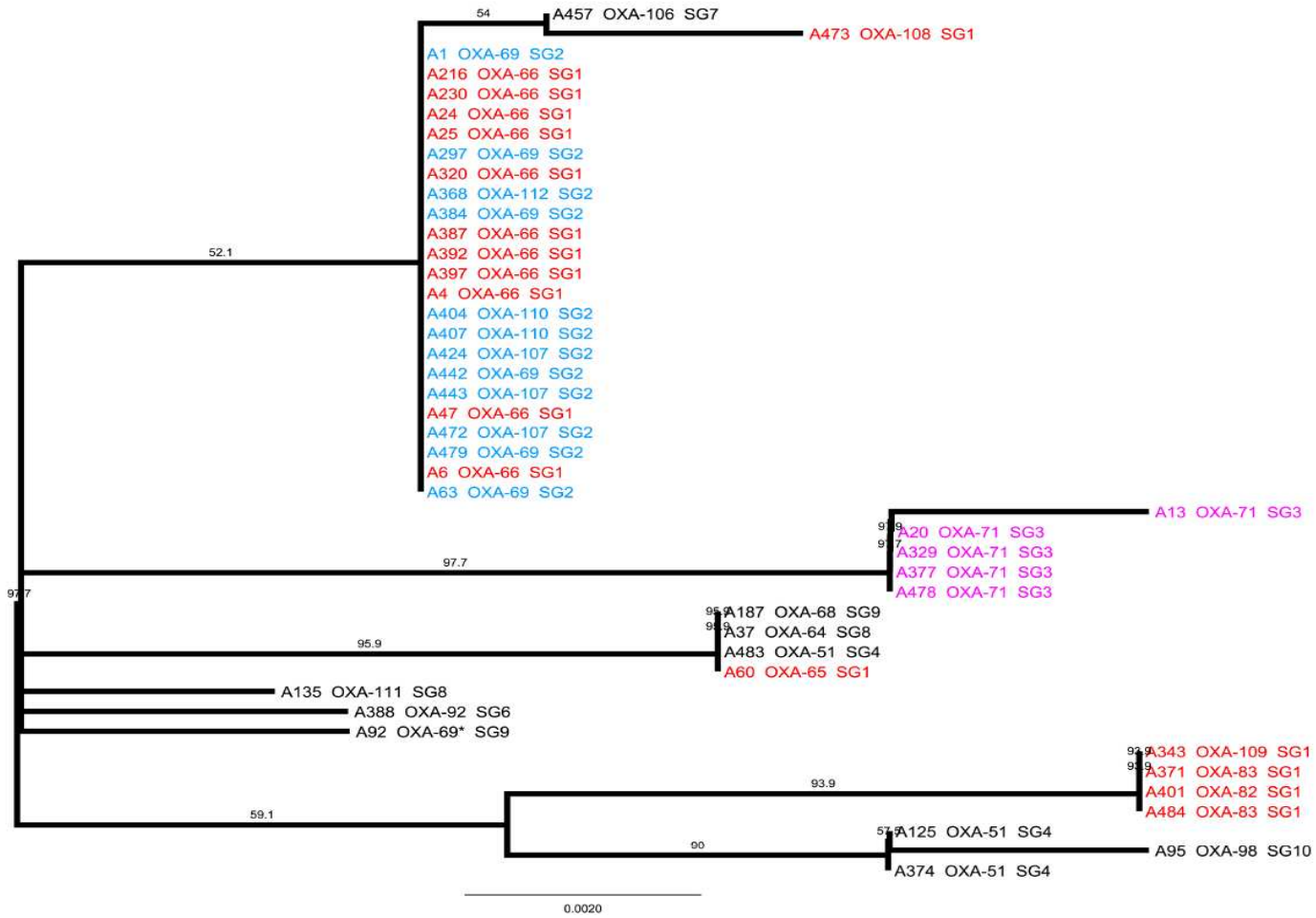

Figure 18: *gyrB* neighbour-joining tree. Isolate names are followed by the OXA-51-like enzyme that they carry and their SG. Branches are labelled with % support. The tree is mid-point rooted. Red, SG1; blue, SG2; pink, SG3.

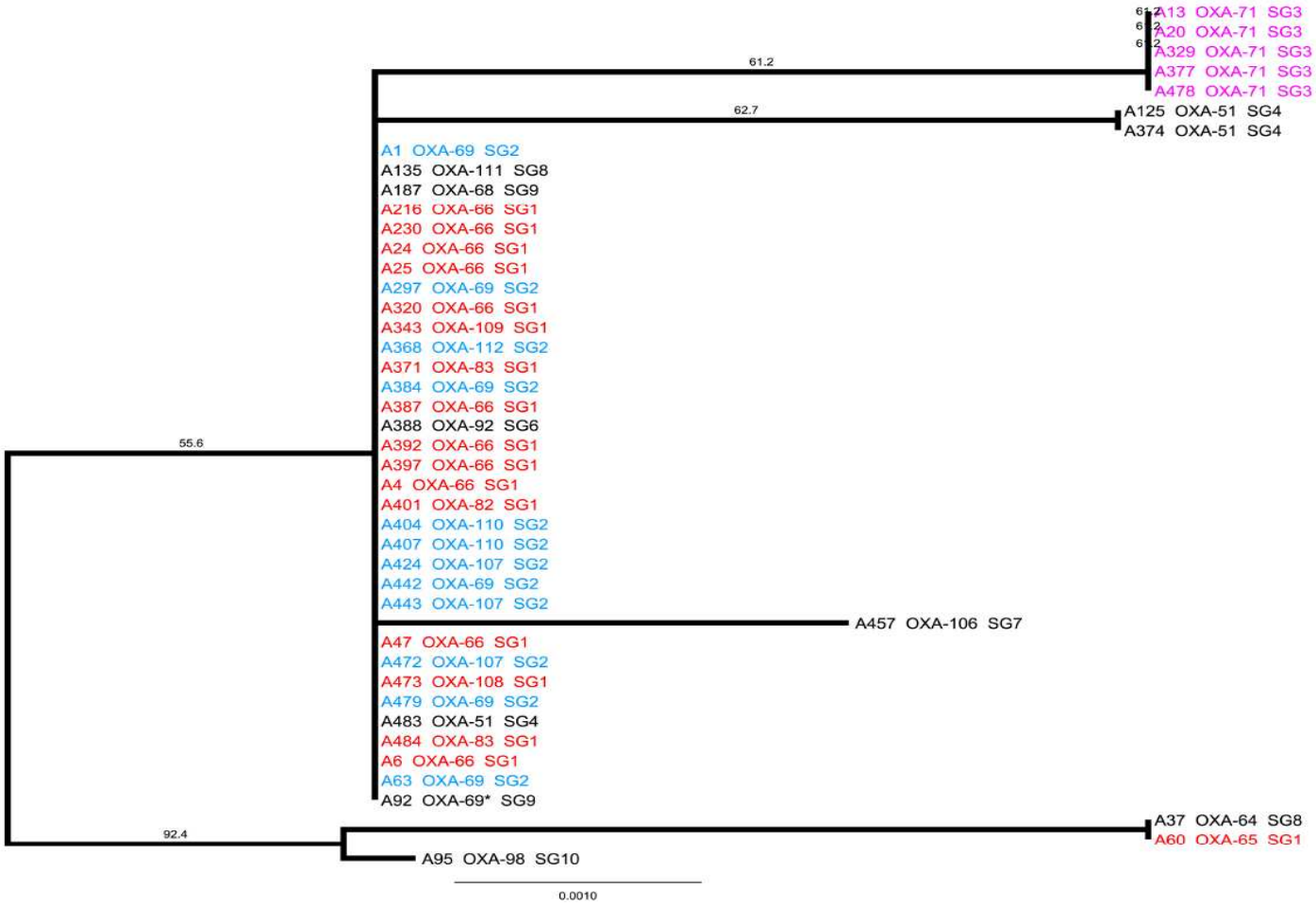

Figure 19: *rpoD* neighbour-joining tree. Isolate names are followed by the OXA-51-like enzyme that they carry and their SG. Branches are labelled with % support. The tree is mid-point rooted. Red, SG1; blue, SG2; pink, SG3.

support shown for the splitting of the two groups. This phylogeny is inconsistent with that for *gpi* as well as those for the other five genes. These visual observations suggest that the *gpi* and *gyrB* loci may not be stable and suitable for use in an MLST scheme.

The visual observations of the differences in the topologies for the *gpi* and *gyrB* phylogenies in relation to those for the other five genes was confirmed via the quartet measure of tree-to-tree distances (Estabrook *et al*, 1985). This method calculates the similarity of two tree topologies to one another by comparing for each possible set of four isolates, whether they are related to one another in the same manner in both trees, or not. Examining the phylogenies for the seven MLST genes demonstrated that for *gyrB* and *gpi* the number of differently resolved quartets was greater than the number of similarly resolved quartets, in contrast to the other five genes (table 13). This demonstrated that the visual observation of inconsistencies was correct. Alleles for all seven genes were analysed with the pairwise homoplasy index (phi) test (Bruen *et al*, 2006). This test compares sites at which there is variation with other 'nearby' sites at which there is variation to determine whether sequences contain a consistent pattern of nucleotide variation. Sudden changes in one sequence from a pattern similar to its closely related sequences to one similar to a more distantly related sequence provides evidence for the sequence having undergone recombination. After analysing the sequences of the alleles for all seven genes the phi test detected statistically significant evidence for recombination ($p = 0.0036$) within the alleles for *gyrB*, but not within the alleles for the other six genes. Together these observations suggest that the *gyrB* and *gpi* genes both have different

Tree comparisons		Q	Same	Different
gyrB	*cpn60*	135751	48678	87073
gpi	*cpn60*	135751	53525	82226
	gyrB	135751	54176	81575
gltA	*cpn60*	135751	72781	62970
	gyrB	135751	48728	87023
	gpi	135751	48678	87073
gdhB	*cpn60*	135751	81976	53775
	gyrB	135751	66570	69181
	gpi	135751	64914	70837
	gltA	135751	79804	55947
rpoD	*cpn60*	135751	85972	49779
	gyrB	135751	66667	69084
	gpi	135751	62436	73315
	gltA	135751	80324	55427
	gdhB	135751	106700	29051
recA	*cpn60*	135751	101700	34051
	gyrB	135751	51342	84409
	gpi	135751	57243	78508
	gltA	135751	81937	53814
	gdhB	135751	93872	41879
	rpoD	135751	92481	43270

Table 13: Quartet measures of tree-to-tree distances. Q, maximum number of resolved quartets; Same, number of quartets that were resolved and the same; Different, number of quartets that were resolved but different.

evolutionary histories to the other five genes, and as such are not reliable or suitable for use within an MLST scheme.

3.5.3 Analysis of concatenated sequences for five loci.

Due to evidence that the *gpi* and *gyrB* loci are unreliable for use in an MLST scheme, further phylogenetic analysis of the isolates in this study was performed using concatenated sequences of the remaining five loci – *gltA, recA, gdhB, cpn60* and *rpoD*. A neighbour-joining tree using these five loci is shown in figure 20.

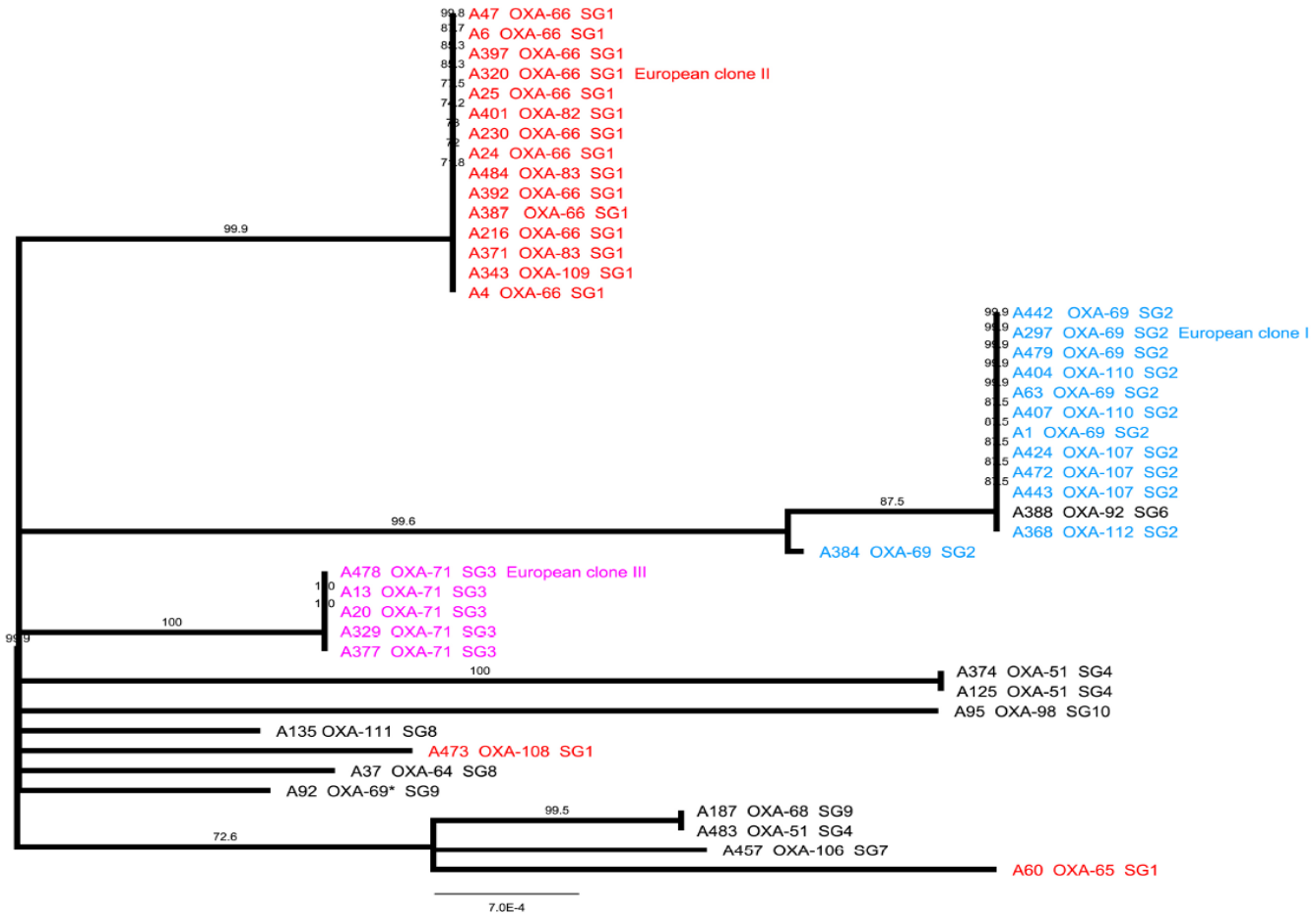

Figure 20: Neighbour-joining tree for 44 isolates using concatenated sequences for five alleles. Isolate names are followed by the OXA-51-like enzyme that they carry and their SG. Branches are labelled with % support. The tree is mid-point rooted. Red, SG1; blue, SG2; pink, SG3.

The phylogeny of the 44 isolates was consistent with SG designations and *bla*$_{\text{OXA-51-like}}$ gene content, and was very well supported. Of the 17 isolates belonging to SG1, 15 were grouped together in the top-most clade. The two SG1 isolates that were not grouped there, A473 and A60, possessed a *bla*$_{\text{OXA-108}}$ and a *bla*$_{\text{OXA-65}}$ gene respectively. Analysis of the individual locus trees indicates that A60 differs from the other SG1 isolates at the *rpoD*, *recA*, *gdhB* and *cpn60* loci, whereas A473 only differs at the *gdhB* and *cpn60* loci.

All of the isolates belonging to SG2 were found in the same clade. Isolate A384 is slightly removed from the other SG2 isolates due to variations within the *cpn60* locus. Isolate A92 that carries a *bla*$_{\text{OXA-69}}$ gene with five silent substitutions compared with the other *bla*$_{\text{OXA-69}}$ sequences, and that belongs to SG9, differed from the SG2 isolates at the *gdhB*, *recA* and *cpn60* loci, and to a lesser extent at the *gltA* locus, confirming that this isolate is very different to the other SG2 isolates despite containing a gene that codes for OXA-69.

The five SG3 isolates were all found together in one clade with 100% support, as these isolates are grouped together in all of the individual locus trees. Two of the SG4 isolates, A374 and A125, were grouped together with 100% support as they were also the same as one another across the five loci. However, the other SG4 isolate, A483, was not grouped with the other two, as it has a different nucleotide sequence for all five loci. As the *bla*$_{\text{OXA-51-like}}$ gene is identical in all three isolates, but the sequences for the alleles at the other five loci are not, it is possible that the *bla*$_{\text{OXA-51}}$ gene in isolate A483 may have been horizontally transferred. The two

isolates that represent SG8 are found on their own separate branches. The isolates differ at the *recA*, *gdhB* and *rpoD* loci, but also possess different OXA-51-like enzymes, OXA-111 and OXA-64, which differ by four amino-acids, and by eight nucleotides. The two isolates representing SG9 are also found on separate branches as they differ at all but the *rpoD* locus. The two OXA-51-like enzymes that these isolates contain, OXA-68 and OXA-69 (with five silent nucleotide substitutions), differ by eight amino-acids and by 12 nucleotides. The fact that some isolates that share a SG, but differ at several MLST loci and carry different $bla_{\text{OXA-51-like}}$ genes suggests that the SG typing scheme may only be of value in discriminating between the three main epidemic lineages containing European clones I, II and III.

3.5.4 Comparisons within and between MLST and $bla_{\text{OXA-51-like}}$ alleles.

To investigate whether the $bla_{\text{OXA-51-like}}$ locus is comparable in terms of diversity to the 'housekeeping' genes used in the MLST scheme, and to other families of β-lactamase genes, the πN/πS measure of diversity was used. All alleles for all seven MLST loci and $bla_{\text{OXA-51-like}}$ genes available at the time were included. In addition, the first 52 different entries on the PubMed website following a search for 'TEM' and 'SHV', and all entries for $bla_{\text{CTX-M-1-like}}$, $bla_{\text{CTX-M-9-like}}$ and bla_{CMY} genes were included (excluding multiples of the same enzyme variant). The πN/πS ratios are shown in table 14. A πN/πS ratio greater than one indicates positive selection i.e. selection for an amino-acid substitution to change the enzyme variant, while a ratio of less than one indicates purifying selection i.e. selection to maintain the enzyme amino-acid sequence.

Locus	Alleles	Pairwise comparisons	π_N	π_S	π_N/π_S
gltA	14	91	0.0072	0.2487	0.0291
gyrB	28	378	0.0087	0.2580	0.0336
recA	16	120	0.0075	0.6157	0.0122
gdhB	20	190	0.0171	0.3049	0.0560
cpn60	17	136	0.0072	0.1657	0.0435
gpi	25	300	0.0093	0.3012	0.0380
rpoD	20	190	0.0146	0.2029	0.0720
bla $_{\text{OXA-51-like}}$	52	1326	0.0108	0.0218	0.2460
bla $_{\text{TEM}}$	52	1326	0.0069	0.0158	0.4357
bla $_{\text{SHV}}$	52	1326	0.0061	0.0151	0.4069
bla $_{\text{CTX-M-1-like}}$	24	276	0.0066	0.0396	0.1664
bla $_{\text{CTX-M-9-like}}$	17	136	0.0048	0.0079	0.6152
bla $_{\text{CMY}}$	20	190	0.0084	0.0480	0.1759

Table 14: πN/πS ratios for MLST genes, *bla*$_{\text{OXA-51-like}}$ genes, and other selected β-lactamase genes.

The πN/πS ratios for the seven MLST 'housekeeping' genes were all very low, and did not differ from one another by more than a factor of 6. However, the *bla*$_{\text{OXA-51-like}}$ alleles produced a πN/πS ratio that was significantly higher than those for the MLST genes ($p < 0.001$), though still considerably less than one, and differed from them by a factor of between 3.4 and 20. This suggests that the *bla*$_{\text{OXA-51-like}}$ genes are under less purifying selection than is seen for purely 'housekeeping' genes (Nei & Gojobori, 1986).

The πN/πS ratios for the selected β-lactamases were also less than one, but again were much greater than those of the 'housekeeping' genes, as expected. Interestingly, the values obtained ranging between 0.16 and 0.62 are in the same range as that obtained for the *bla*$_{\text{OXA-51-like}}$ alleles, suggesting that the *bla*$_{\text{OXA-51-like}}$ genes are under a similar level of selection to other major groups of β-lactamase genes.

3.6 Dose response curves.

To determine if the presence of the intrinsic *bla*$_{\text{OXA-51-like}}$ genes in *A. baumannii* increased the ability of the species to survive carbapenem pressure, even if the isolates themselves are not resistant, dose response curves were calculated. The comparison of curves for three *A. baumannii* isolates, two genospecies 13TU isolates and one genospecies 3 isolate is shown in figure 21. The *A. baumannii* isolate ATCC 19606 was used as a sensitive reference strain, while isolates A24 and A343 were chosen as they are sensitive representatives of the highly prevalent sequence group 1. For *A. baumannii* isolates ATCC19606, which has no IS*Aba1* elements, and A24 which has IS*Aba1* elements but not upstream of the *bla*$_{\text{OXA-51-like}}$ gene, there was no major difference in their ability to survive meropenem challenge compared with the genospecies 3 and 13TU isolates. This indicates that the presence of the *bla*$_{\text{OXA-51-like}}$ gene alone, without the presence of the IS*Aba1* sequence to potentially enhance its expression, does not greatly affect the survivability of the isolate. In contrast isolate A343, which has an IS*Aba1* sequence upstream of its *bla*$_{\text{OXA-51-like}}$ gene, is much better able to survive meropenem challenge than the other isolates. Despite the fact that the meropenem MIC for this isolate is only 4 mg L^{-1} (table 1), considered of intermediate susceptibility by BSAC 2008 criteria and susceptible by Clinical and Laboratory Standards Institute (CLSI) 2006 criteria, the isolate still maintains ≥100% viability at a meropenem concentration of 14.6 mg L^{-1} (equation of the line between points at 8 and 16 mg L^{-1} is $y = -37.202x + 645.89$), and is not reduced to a viability of less than 10% until the meropenem concentration reaches 45.8 mg L^{-1} (equation of the line between points at 32 and 64 mg L^{-1} is $y = -0.5158x + 33.633$). This suggests that the potential of IS*Aba1* to increase expression of the *bla*$_{\text{OXA-51-like}}$ gene in isolate

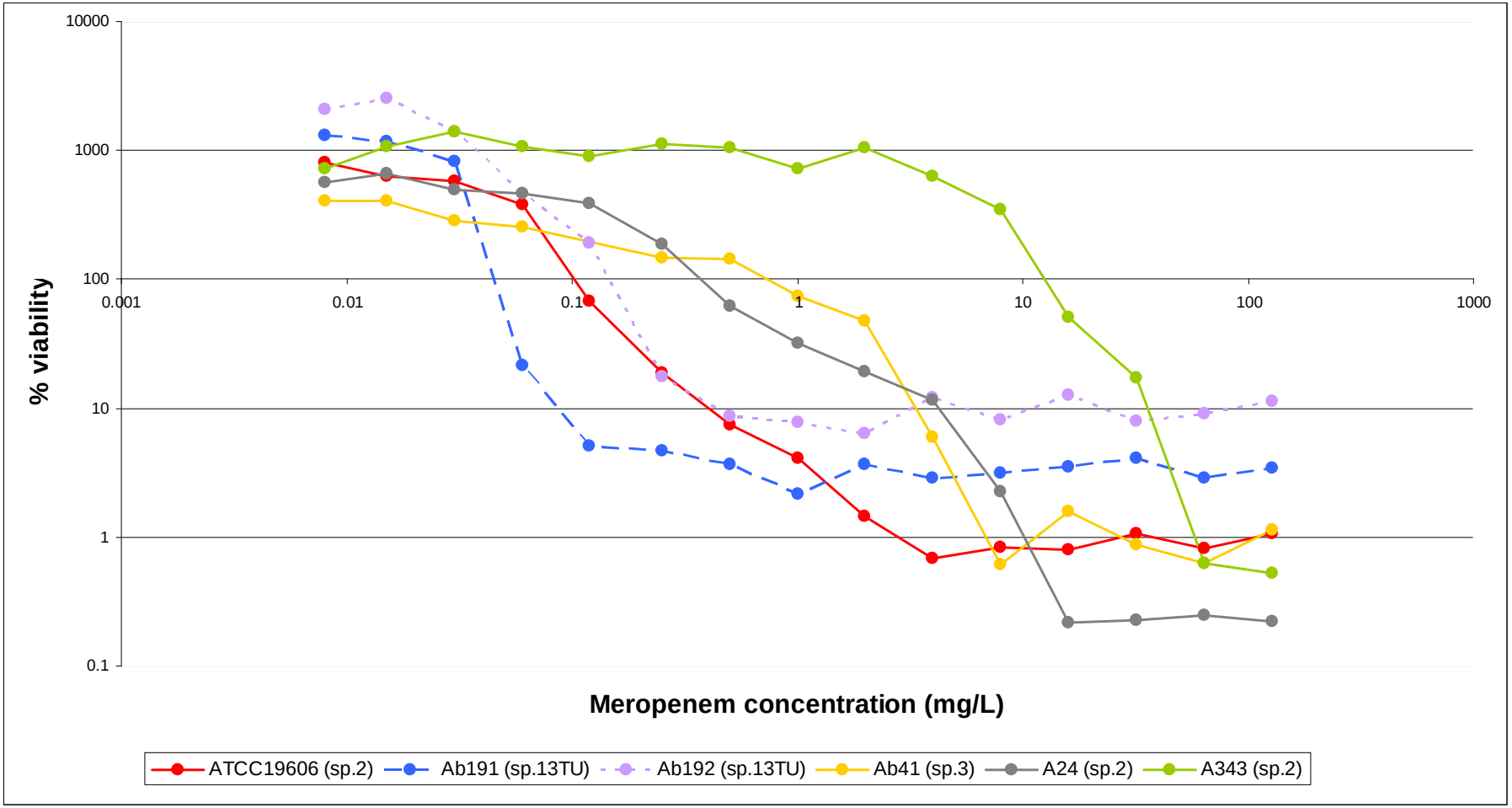

Figure 21: Percentage viability of *Acinetobacter* species after 3 hours challenge with meropenem.

A343 may allow it to survive meropenem challenge much better than the other isolates, with viability of ≥100% maintained at a concentration approaching 4-fold the isolates MIC value. However, it should be noted that due to the limitations of the isolate collection available, the three *A. baumannii* isolates included in this experiment all possess different $bla_{\text{OXA-51-like}}$ genes (ATCC 19606 carries $bla_{\text{OXA-98}}$, A24 carries $bla_{\text{OXA-66}}$ and A343 carries $bla_{\text{OXA-109}}$), and it is possible that these different enzyme variants may be contributing to the different responses to meropenem that were observed.

3.7 Mutation study.

In order to determine whether sensitive *A. baumannii* can acquire resistance to the carbapenems conferred by the $bla_{\text{OXA-51-like}}$ genes under selective pressure, a mutation study was conducted. Isolates were grown under increasing concentrations of meropenem, and mutants examined for changes to the gene sequence, or for the insertion of IS*Aba1* upstream of the gene.

The increasing concentrations of meropenem that growth was detected on for the five studied isolates are shown in figure 22. Isolate ATCC 19606 was included as the sensitive type strain, and the other four isolates were included to represent each of the three major sequence groups, as well as an isolate carrying $bla_{\text{OXA-65}}$, encoding the enzyme that appears to be the closest to the centre of the OXA-51-like functional map (see section 3.4) and therefore present the greatest potential to acquire substitutions resulting in a structure more similar to one of the most prevalent enzymes.

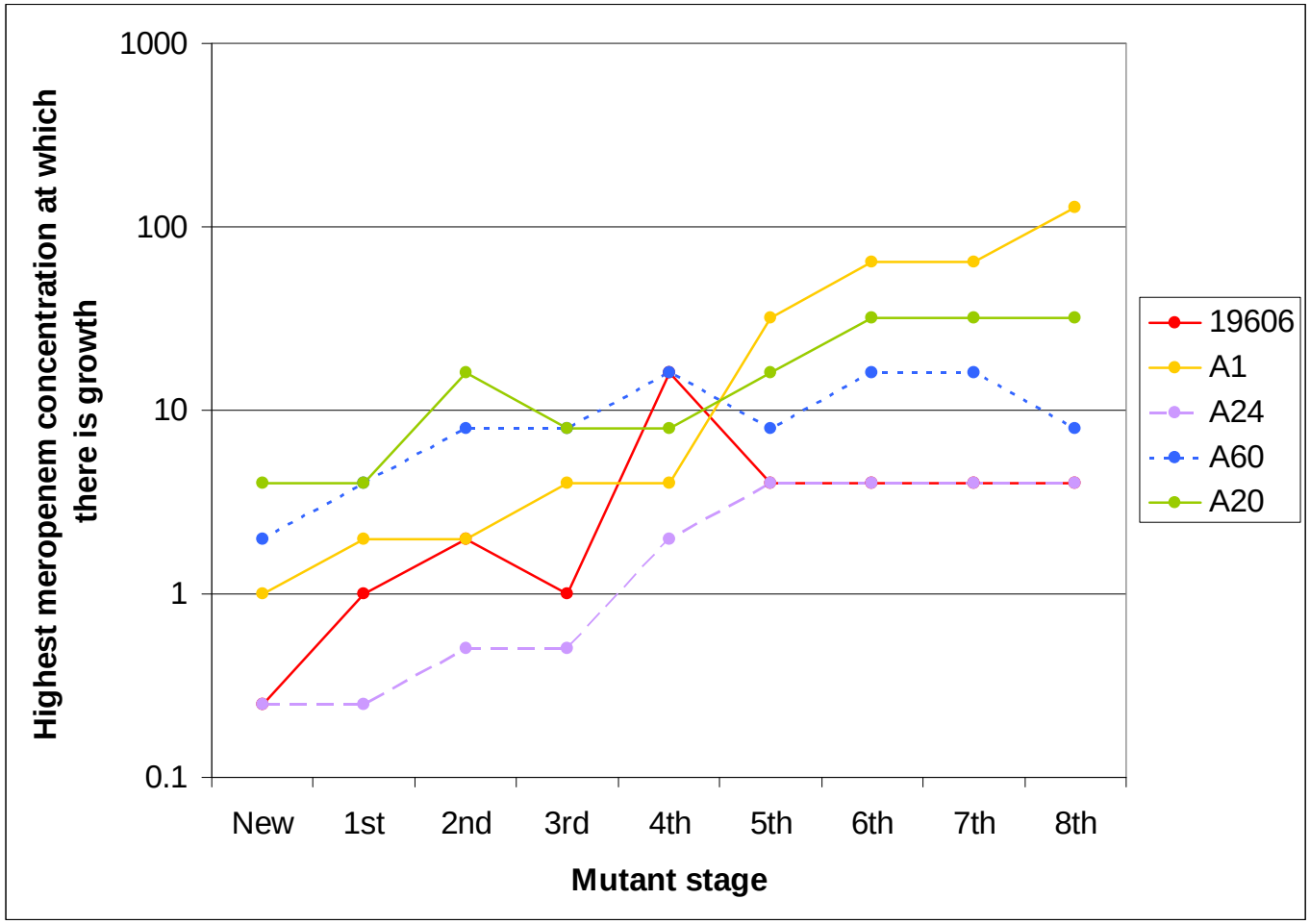

Figure 22: The highest concentrations of meropenem on which successive cultures of isolates were able to grow. Concentrations are in mg L^{-1}.

Isolate A1 (representing SG2 carrying *bla*$_{\text{OXA-69}}$) demonstrated the greatest fold-increase in the concentration of meropenem which it could grow on, increasing from 1 mg L^{-1} to 128 mg L^{-1}. The type strain ATCC19606 and isolate A24 (representing SG1 carrying *bla*$_{\text{OXA-66}}$) showed the next greatest increase, going from 0.25 mg L^{-1} to 4 mg L^{-1}, a 16-fold increase. Isolates A60 (representing SG1 carrying *bla*$_{\text{OXA-65}}$) and A20 (representing SG3 carrying *bla*$_{\text{OXA-71}}$) produced an 8-fold increase, going from 2 and 4 mg L^{-1} to 16 and 32 mg L^{-1} respectively. Sequencing of the *bla*$_{\text{OXA-51-like}}$ genes of the mutant isolates demonstrated that there were no nucleotide changes in any of the genes in relation to their parent isolates, and in the isolates that possess IS*Aba1* (A60 and A24), the insertion sequence had not moved to insert upstream of the *bla*$_{\text{OXA-51-like}}$ gene.

Analysis of MIC data obtained for the parent and mutant isolates eight months after their isolation is shown in table 15. The meropenem MICs for the A1 mutant and A20 mutant are 8-fold lower than the concentration of meropenem on which they were isolated. However, the MICs for four of the parent isolates (ATCC19606, A1, A20, and A60) had also decreased from those shown in tables 7 and 8, and previously determined for ATCC19606. This suggests that the mechanism of increased resistance in these isolates is transient and gradually decreases over time that the isolate is stored. However, the MICs for the mutants of isolates ATCC19606, A24 and A60 are at the same concentration as that of the meropenem plate they were isolated from. Interestingly, the increases in resistance to imipenem in the mutant isolates were much lower than the increases in resistance to meropenem, suggesting

Isolate	Imipenem		Meropenem	
	MIC	Fold-increase	MIC	Fold-increase
ATCC19606	0.06		0.12	
ATCC19606 M	0.5	8	4	32
A1	0.12		0.25	
A1 M	0.5	4	16	64
A20	0.12		0.5	
A20 M	0.5	4	4	8
A24	0.12		0.25	
A24 M	1	8	4	16
A60	0.25		0.5	
A60 M	1	4	16	32

Table 15: MICs of imipenem and meropenem for parent and mutant isolates.

M, mutant isolate. MICs are in mg L^{-1}.

that the mechanism of increased resistance that was being selected by meropenem was not as effective at providing resistance to imipenem.

3.8 Growth study.

In order to investigate the degree of heterogeneity that can occur in *A. baumannii* pulsed-field gel electrophoresis patterns and the *bla*$_{\text{OXA-51-like}}$ gene over a medium-term period of time, a growth study was conducted. Isolate A418 was studied as it represents the most prevalent sequence group, SG1, and carries the most prevalent *bla*$_{\text{OXA-51-like}}$ gene, *bla*$_{\text{OXA-66}}$. Additionally, this isolate possess IS*Aba1*, though it is not found to be present upstream of the *bla*$_{\text{OXA-66}}$ gene. Therefore this experiment would also examine whether long-term exposure to sub-inhibitory concentrations of meropenem could induce the movement of the insertion sequence to insert upstream of the *bla*$_{\text{OXA-66}}$ gene.

Isolate A418 was grown for 100 days continually in nutrient broth, and for 100 days continually in nutrient broth containing imipenem at 1/8th of the isolate's MIC.

Analysis of the PFGE patterns generated for the parent isolate and for the two 100 day-old isolates, shown in figure 23, shows that the isolate grown in nutrient-rich conditions without any pressures had an identical PFGE pattern to the parent isolate. However, the isolate grown under a sub-inhibitory concentration of imipenem had a slightly different pattern with 88% similarity to the parent isolate, and differing by three bands. These differences are not major, and the isolates would all be considered members of the same epidemic strain using a cut-off of ≥87%, though only marginally (Seifert *et al*., 2005). These data demonstrate that the PFGE pattern for isolate A418 can vary by almost enough for the isolate to be classified as a different epidemic strain after only 100 days growth in stressful conditions, and this may explain why PFGE analysis in section 3.2 was unable to group isolates in agreement with other typing methods. Sequencing of the *bla*$_{\text{OXA-51-like}}$ genes of the two 100 day-old isolates revealed identical sequences to the parent isolate, and in neither isolate had the IS*Aba1* element in this isolate inserted upstream of the *bla*$_{\text{OXA-51-like}}$ gene.

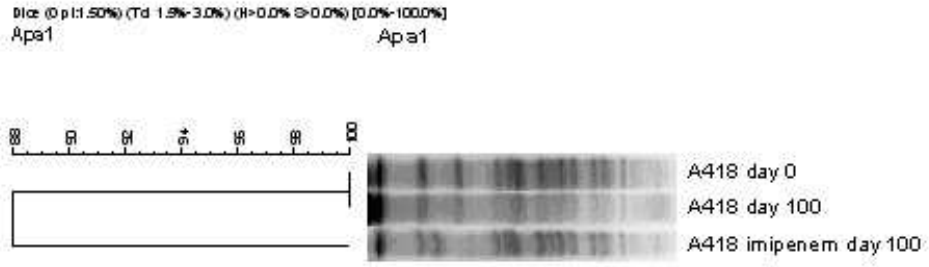

Figure 23: Pulsed-field gel electrophoresis analysis of the original parent isolate, and the two 100 days-old isolates.

3.9 *bla*$_{\text{OXA-51-like}}$ sequence analysis.

3.9.1 Bayesian analysis of the species *A. baumannii*.

Analysis of the *bla*$_{\text{OXA-51-like}}$ genes of the 64 isolates included in this study has shown that certain groups of closely related genes are associated with particular lineages within the species. Investigation of the phylogeny of the *bla*$_{\text{OXA-51-like}}$ genes and how this relates to that of the isolates that carry them was conducted through bayesian analysis of the nucleotide sequences.

A phylogeny for *A. baumannii* was constructed using the sequences for the four 'housekeeping' genes *gltA*, *recA*, *cpn60* and *rpoD* previously amplified for the 44 isolates in this study. Additionally, the sequences for these genes were downloaded from the PubMed website for the six published *A. baumannii* genome sequences (ACICU, AB0057, AB307-0294, AYE, SDF and ATCC17978), and for two genospecies 13TU isolates which were used as outgroups. The *gpi* and *gyrB* genes that it was previously shown demonstrated evidence for horizontal gene transfer were not used, and neither was *gdhB* as this gene was not found in isolate SDF, and therefore it can not be relied upon as a conserved 'housekeeping' gene. The resulting phylogeny is shown in figure 24. As was observed previously with the MLST data, all of the isolates with similar *bla*$_{\text{OXA-51-like}}$ genes were grouped together, with isolates belonging to SG1 found in the top clade of the tree, all of the SG2 isolates in the large central clade of the tree, and the five SG3 isolates in the bottom half of the tree, with all clades diverging independently from a common root for the species. Interestingly the main clades are not all found at the same distance from the common root. For example, the SG1 isolate A397 is at a distance of 0.2146 from the root,

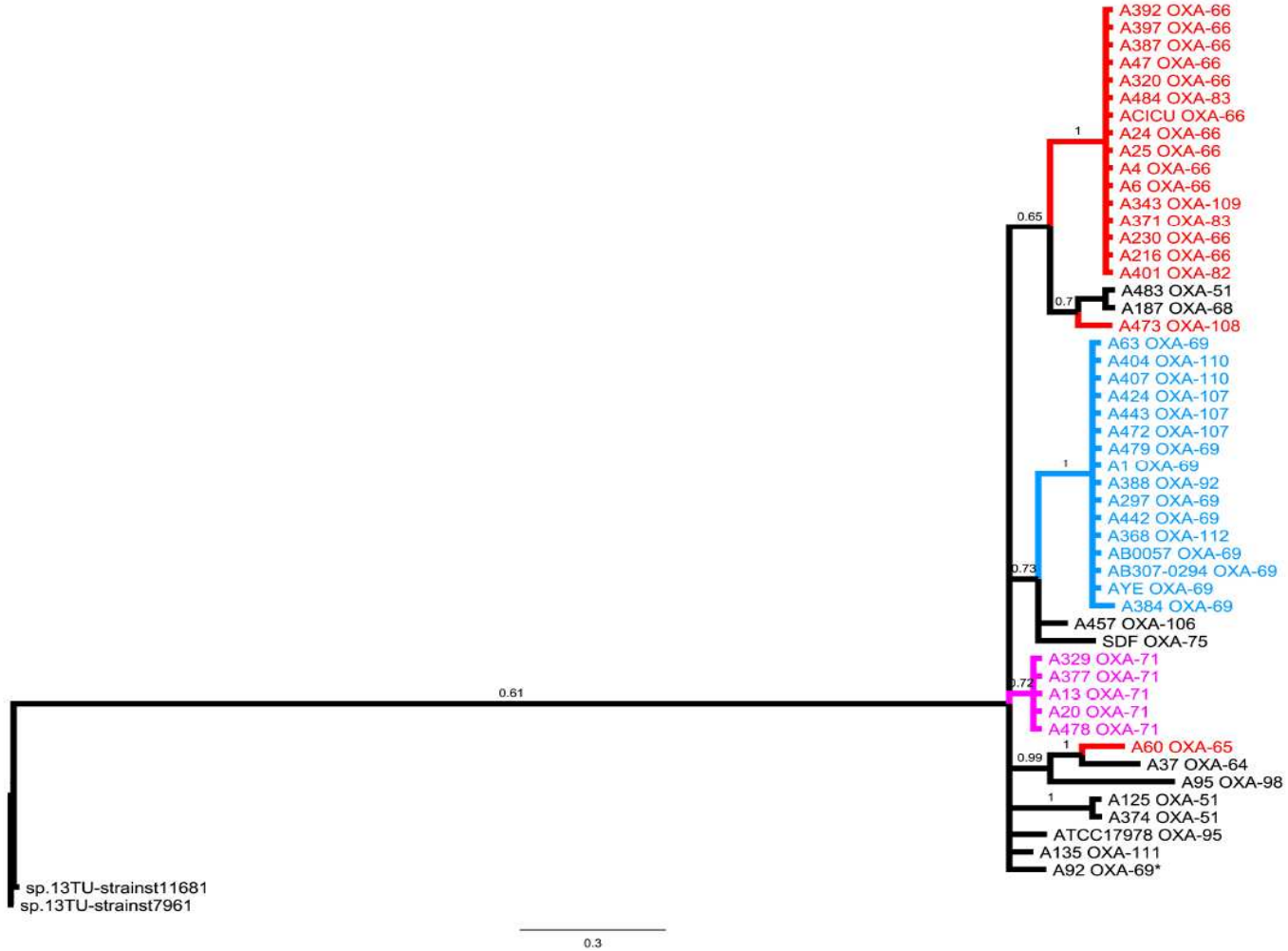

Figure 24: Bayesian phylogeny of *A. baumannii*. Branch tips are labelled with isolate names and the OXA-51-like enzyme they contain. Branches are labelled with posterior probabilities. The tree is rooted to sp.13TU strain st7961. Red, SG1; blue, SG2; pink, SG3.

indicating a sequence divergence of 21% from the root. On the other hand, a branch length of 0.1906 for the SG2 isolate A479 represents 19% sequence divergence, and the length of 0.0183 for the SG3 isolate A13 represents 1.8% sequence divergence from the root.

3.9.2 Bayesian analysis of *bla*$_{\text{OXA-51-like}}$ genes.

To estimate the phylogeny of the *bla*$_{\text{OXA-51-like}}$ genes, all available allele variants were downloaded from the PubMed website. Added to these were allele variants identified from isolates from Pakistan not included in this study, and variants identified by Dr. Alsultan in our laboratory (personal communication). A bayesian analysis of all of these alleles is shown in figure 25.

The *bla*$_{\text{OXA-51-like}}$ genes are believed to be intrinsic and ubiquitous in *A. baumannii*, and this study has shown the close relationship between groups of similar *bla*$_{\text{OXA-51-like}}$ genes and certain lineages within the species. The hypothesis that the *bla*$_{\text{OXA-51-like}}$ genes have been co-evolving with the *A. baumannii* isolates that carry them with little horizontal gene transfer was used as a basis for rooting the *bla*$_{\text{OXA-51-like}}$ phylogeny in the absence of an outgroup for these enzymes. Therefore the *bla*$_{\text{OXA-51-like}}$ tree was rooted at the same position as that in the *A. baumannii* species tree in figure 24.

The tree is split into seven main clades, with the different groups of *bla*$_{\text{OXA-51-like}}$ genes clustered in separate clades. All of the clades that carry the different enzyme groups diverge independently from a common root. However, the different clades do

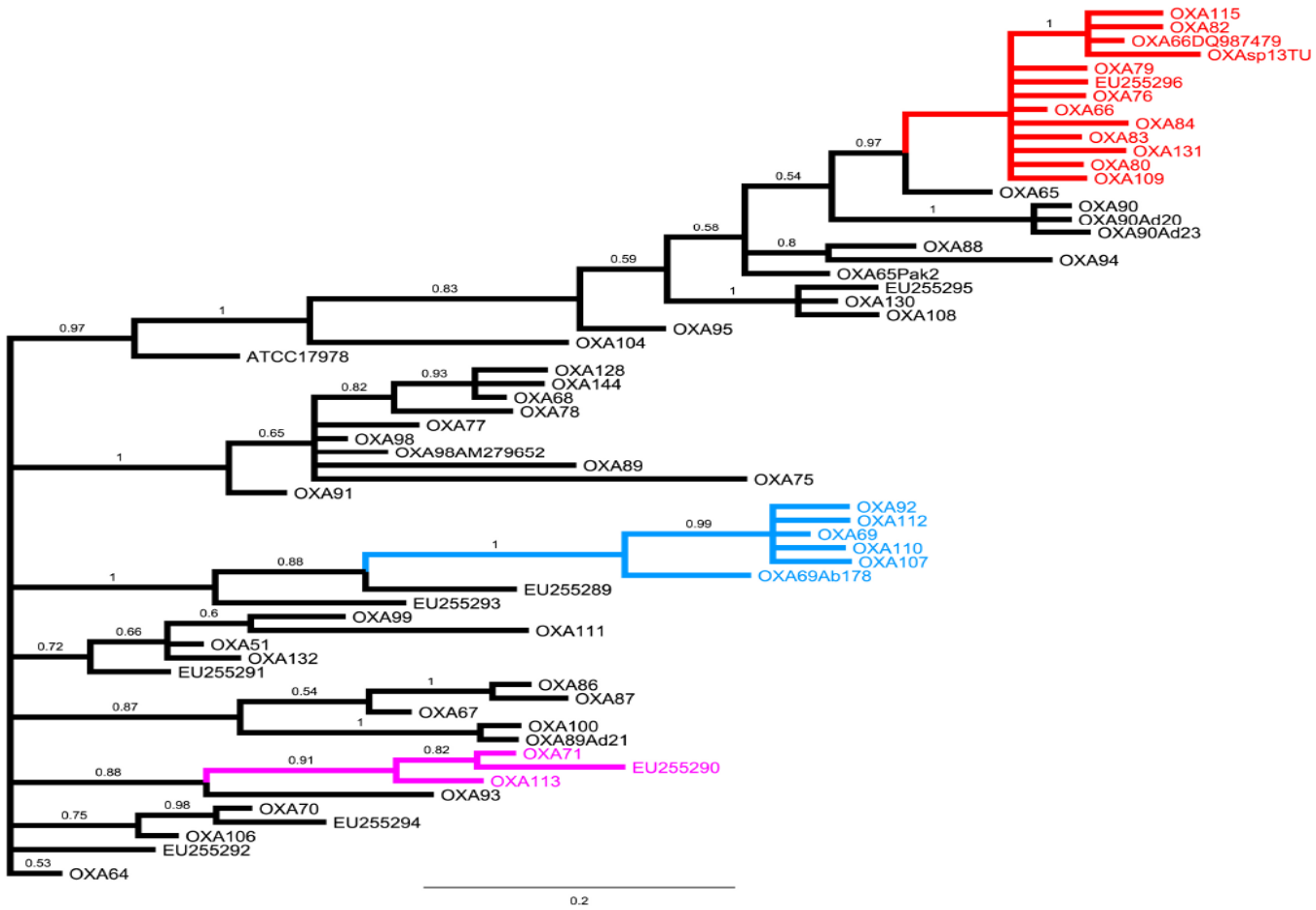

Figure 25: Bayesian phylogeny of all available *bla*$_{\text{OXA-51-like}}$ allele variants. Branch tips are labelled with the enzyme name. For unnamed enzymes the PubMed accession number is used. For variants of named enzymes the enzyme name is followed by an accession number or isolate name as appropriate. Branches are labelled with posterior probabilities. The tree is rooted in concordance with figure 15. Red, *bla*$_{\text{OXA-66-like}}$ genes; blue, *bla*$_{\text{OXA-69-like}}$ genes; pink, *bla*$_{\text{OXA-71-like}}$ genes.

not all extend the same distance from the root. The enzyme OXA-66 extends a distance of 0.6674 from the root, representing a sequence divergence of 67%. The enzyme OXA-69 has a shorter branch length of 0.5152 translating to 52% sequence divergence, while OXA-71 is at a distance of only 0.3255, diverging 33% from the root.

The top-most clade of the tree contains *bla*$_{\text{OXA-66}}$ and related genes at its tip, and extends the furthest from the root. The ladder-like topology of the clade is indicative of recurrent selective sweeps over a period of time, a classic example of which is seen in the topology of trees examining the haemagglutinin gene in human influenza A virus strains (Cobey and Koelle, 2008). A similar pattern is seen in the clade encompassing *bla*$_{\text{OXA-69}}$ and similar genes. Other smaller clades that don't extend as far from the root also show a topology indicative of selective sweeps. However, this is not so evident in the clade containing *bla*$_{\text{OXA-98}}$. This clade is much more compact with many of the genes emerging from the same node. The two clades in which the ladder-like topology is most pronounced (the OXA-66-like and OXA-69-like enzymes) are also the two clades with the longest branch lengths.

3.9.3 Comparison of *A. baumannii* and *bla*$_{\text{OXA-51-like}}$ phylogeny.

A direct comparison of the phylogenies for the *bla*$_{\text{OXA-51-like}}$ genes and *A. baumannii* is shown in figure 26. There is a large degree of similarity in the topologies of the two trees, which would be expected if the *bla*$_{\text{OXA-51-like}}$ genes have been evolving within *A. baumannii* for a long period of time. The concordance of the two trees is good evidence for the co-evolution of the *bla*$_{\text{OXA-51-like}}$ genes and the 'housekeeping'

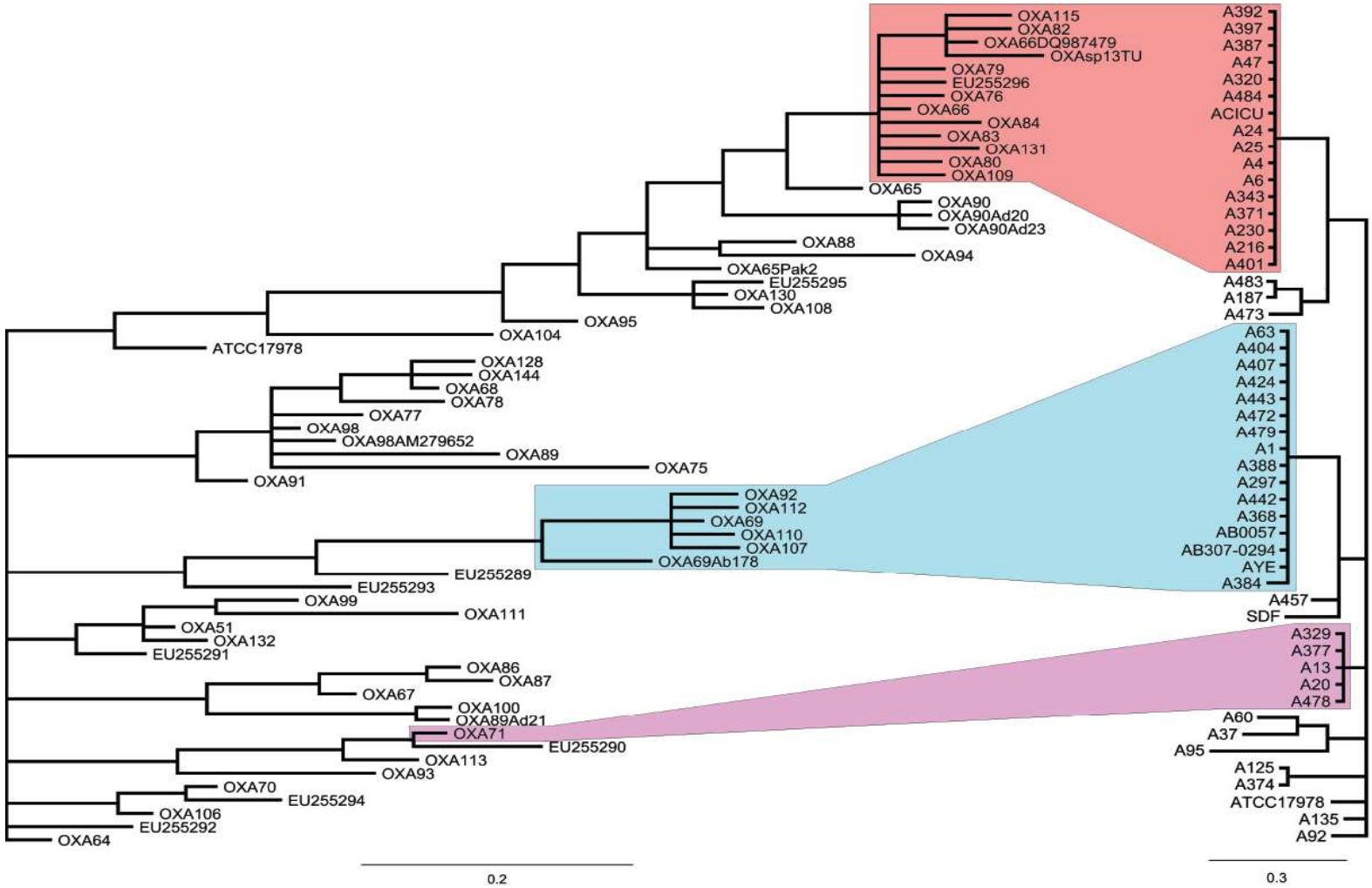

Figure 26: Comparison of bayesian phylogenies for *bla*$_{\text{OXA-51-like}}$ genes and *A. baumannii*. Boxes encompass *bla*$_{\text{OXA-51-like}}$ genes associated with SG1 (red), SG2 (blue) and SG3 (pink) on the left, and isolates allocated to SG1, 2 and 3 on the right.

genes. However, there are some notable exceptions. As previously noted in the MLST analysis in section 3.5.3, the three isolates that contain a *bla*$_{\text{OXA-51}}$ gene (A483, A125 and A374) are separated in the *A. baumannii* tree. Additionally, isolate A135 is not clustered with the SG4 isolates, despite the fact that the *bla*$_{\text{OXA-111}}$ gene that it possesses is found within the same clade as *bla*$_{\text{OXA-51}}$. Isolate A60 that belongs to SG1 and carries *bla*$_{\text{OXA-65}}$ is separated from the other SG1 isolates, and is grouped with A37, which carries *bla*$_{\text{OXA-64}}$.

The similarities in the two phylogenies are most clearly demonstrated when examining the clusters of enzymes and isolates associated with the three major epidemic lineages associated with SG1, 2 and 3. Measurements of the branch lengths for *bla*$_{\text{OXA-66}}$, *bla*$_{\text{OXA-69}}$ and *bla*$_{\text{OXA-71}}$, along with those for three representative isolates that carry these genes are shown in table 16. For both the *bla*$_{\text{OXA-51-like}}$ genes and for *A. baumannii* as a species, SG1-associated *bla*$_{\text{OXA-51-like}}$ genes and isolates are evolving at the fastest rate, SG2-associated genes and isolates are evolving at the second fastest rate, with SG3-associated genes and isolates evolving most slowly of the three groups. Interestingly, in all cases the *bla*$_{\text{OXA-51-like}}$ genes are evolving at a faster rate than the isolates that possess them. This demonstrates that the *bla*$_{\text{OXA-51-like}}$ genes are evolving faster than the rest of the *A. baumannii* genome, as would be expected if the ‘housekeeping’ genes were under a higher degree of purifying selection than the *bla*$_{\text{OXA-51-like}}$ genes, as demonstrated by the $\pi N/\pi S$ ratios calculated in section 3.5.4.

SG	OXA	Branch length	Rate	Isolate	Branch length	Rate
1	66	0.6674	36.47	A230	0.2146	11.73
2	69	0.5152	28.15	A479	0.1906	10.42
3	71	0.3255	17.79	A13	0.0183	1

Table 16: Branch lengths and mutation rates for *bla*$_{\text{OXA-51-like}}$ genes and isolates that possess them. Rate, the relative evolutionary rate as a function of the shortest branch length of A13.

3.10 OXA-51-like structural models.

3.10.1 Identification of regions of variance.

While there is a significant degree of sequence diversity within the *bla*$_{\text{OXA-51-like}}$ genes, the majority of nucleotide changes are likely to be synonymous, or to result in an amino-acid change that has little impact on enzyme function. In order to identify regions of the OXA-51-like enzymes where variation may affect the enzyme function, the percentage amino-acid variation at all sites within the enzymes was compared with the $\pi N/\pi S$ ratios across 50-nucleotide-wide windows within the gene sequences (figure 27).

The data in figure 27 was used to identify a number of sites at which there was a one or more of the following criteria: a large amount of amino-acid variation (>10%), a $\pi N/\pi S$ ratio greater than one, and proximity to a region identified from the OXA-40 crystal structure as being involved in enzyme activity or carbapenem specificity. These residues would be taken forward for further investigation. Due to the limitations of the structural models produced for the OXA-51-like enzymes, only residues from number 30 onwards could be investigated. This process resulted in the identification of 12 sites of interest: positions 36, 57, 107, 117, 129, 130, 146, 156, 167, 168, 194 and 225. For each of these sites, two structural models that vary at the

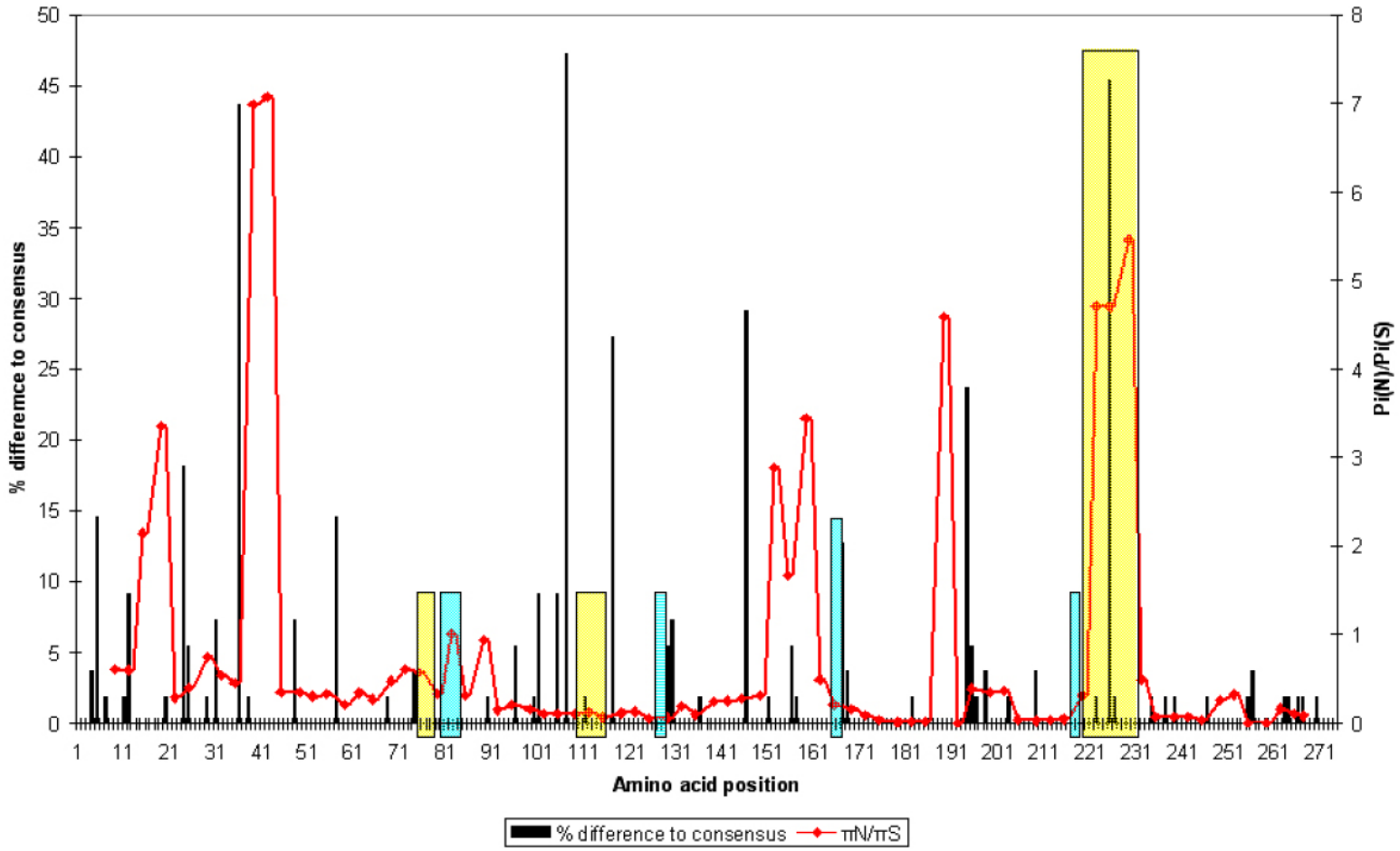

Figure 27: OXA-51-like % amino-acid differences to consensus sequence compared with *bla*$_{\text{OXA-51-like}}$ πN/πS ratios.
Blue boxes indicate regions thought to be active site elements and yellow boxes indicate regions thought to be involved in carbapenem specificity, based upon the OXA-40 crystal structure.

appropriate position were examined to identify the potential impact of the amino-acid changes.

3.10.2 Examination of structural models.

3.10.2.1 Positions 36, 57, 107, 117, 146, 156 and 194.

Of the 12 sites that were examined, 7 contained amino-acid changes the effects of which could not be determined from the structural models available. Positions 36, 57, 107, 117, 146, 156 and 194 were not found near the active site cleft, and were found on, or in the case of position 57 very near, the enzyme surface. In most cases the different amino-acids found at each site did confer different properties, as shown in table 17. The majority of these enzyme variations did not appear to have an effect on the structure of the enzyme.

Amino-acid position	Amino-acid	Properties
36	Glutamate	Negative, polar
	Lysine	Positive, hydrophobic, polar
	Aspartate	Negative, polar
	Valine	Hydrophobic, aliphatic
57	Glutamine	Polar
	Histidine	Positive, aromatic, hydrophobic, polar
	Arginine	Positive, polar
107	Lysine	Positive, hydrophobic, polar
	Glutamine	Polar
	Glutamate	Negative, polar
117	Aspartate	Negative, polar
	Asparagine	Polar
146	Lysine	Positive, hydrophobic, polar
	Asparagine	Polar
156	Alanine	Hydrophobic
	Threonine	Hydrophobic, polar
194	Glutamine	Polar
	Proline	Aliphatic, hydrophobic
	Leucine	hydrophobic aliphatic

Table 17: Properties of amino-acids found at seven positions.

In addition to the different properties of the various enzymes, at position 57 the histidine that is found in enzymes such as OXA-69 forms a hydrogen bond with the glutamine at position 62. In enzymes such as OXA-66 that have a glutamine at position 57, this hydrogen bond does not exist. This may have a subtle effect of the structure of the enzyme. At position 194 enzymes such as OXA-51 have a proline residue, whereas enzymes such as OXA-66 and OXA-69, from the successful SG1 and SG2 lineages, have a glutamine residue (see appendix A for alignments). Position 194 is found just at the start of helix α8 and forms part of the loop between helix α7 and helix α8 that turns the latter helix back into the enzyme structure (figure 28). Due to the properties of proline and its ability to form stable tight direction changes in molecular structures, it is likely that variation between proline and glutamine at position 194 has a significant effect on enzyme structure, though this could not be visualised due to the limitations of the models based upon the OXA-40 backbone.

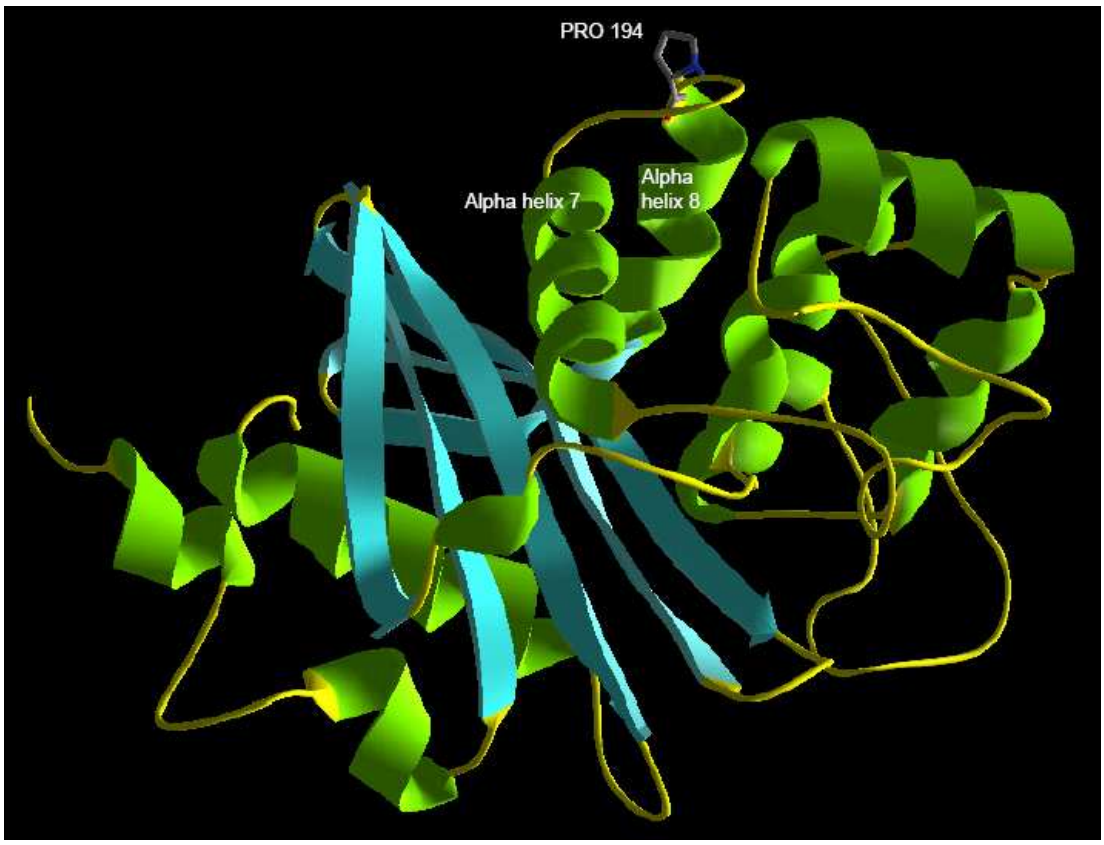

Figure 28: Proline residue at position 194 within the turn between helices α7 and α8 in OXA-51. α-helices are in green, β-sheets are in blue, loops are in yellow. Structure was visualised in DeepView.

3.10.2.2 Position 129.

Residues at position 129 are found at the end and bottom of the active site cleft, not too far from the active site serine at position 80. Enzymes such as OXA-66 have an isoleucine at position 129, which sits just to the side of the base of the cleft, 5.73 Å distant from the active site serine (figure 29). In some enzymes such as OXA-83, one of the potential more recently evolved SG1 enzymes, the isoleucine is substituted for a leucine residue. The leucine residue sits slightly closer to the active site serine at a distance of 5.48 Å, and takes up more space along the bottom of the active site cleft than the isoleucine residue does (figure 25). This change in shape within the active

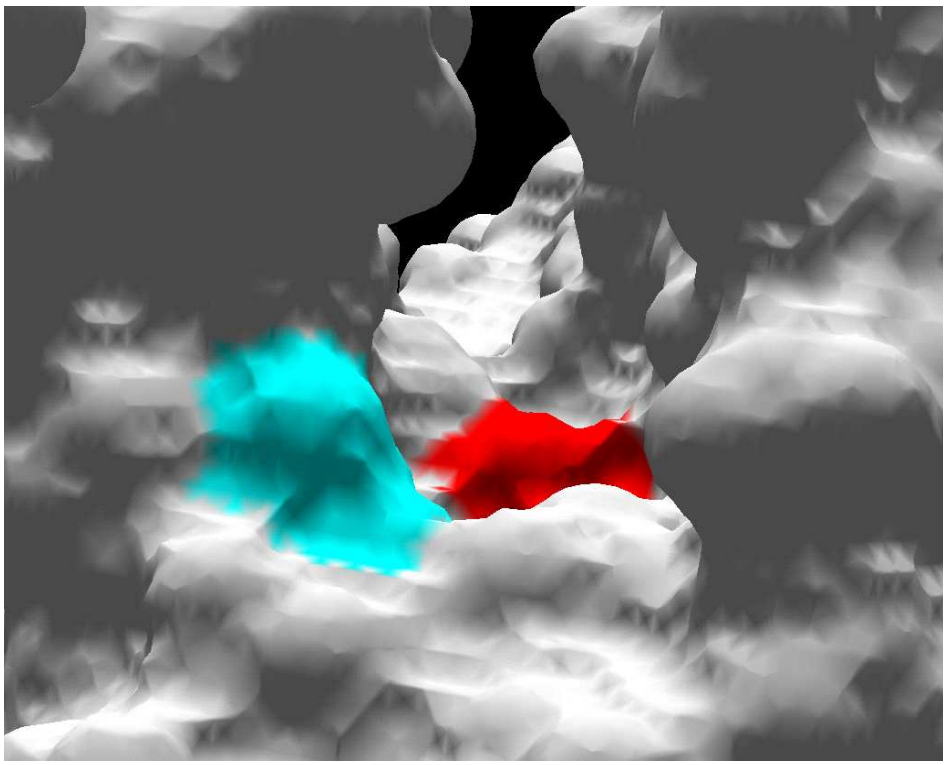

Figure 29: Molecular surface diagram showing view down the active site cleft of OXA-66 (grey) with the active site serine 80 (red) and an isoleucine at position 129 (blue) indicated. Structure was visualised in DeepView.

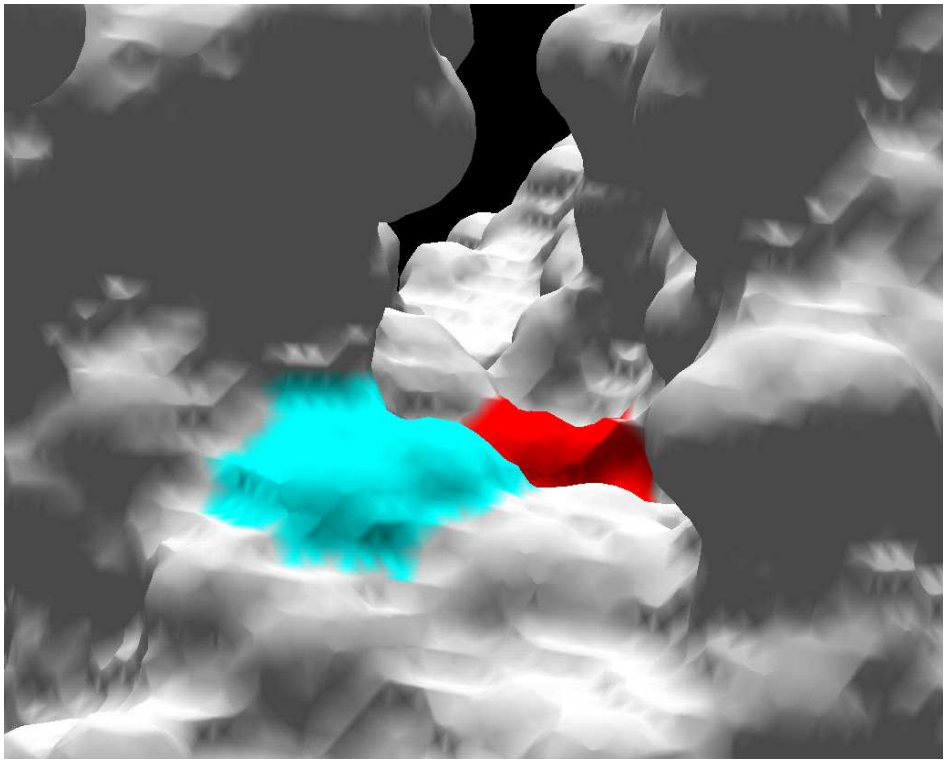

Figure 30: Molecular surface diagram showing view down the active site cleft of OXA-83 (grey) with the active site serine 80 (red) and a leucine at position 129 (blue) indicated. Structure was visualised in DeepView.

site cleft reduces the amount of space for substrate molecules, and as such may affect the substrate profile or rate of hydrolysis of the enzymes. Amino-acid substitutions at position 129 occur independently in the *bla*$_{\text{OXA-66}}$ and the *bla*$_{\text{OXA-69}}$ clades of the *bla*$_{\text{OXA-51-like}}$ phylogeny, suggesting that there is a degree of positive selection for this variation.

3.10.2.3 Position 130.

Variations in the amino-acid at position 130 adjacent to those that occur at position 129 are also observed. Enzymes such as OXA-66 have a proline residue that is likely to provide stability for the isoleucine residue at position 129, as well as for the hydrogen bond between residues alanine 128 and valine 131 (figure 31). In other enzymes such as OXA-115, a potential more recently evolved SG1 enzyme, the proline at position 130 is substituted for a leucine residue (figure 32). This change is likely to result in movement of the isoleucine residue at position 129, and may also affect the hydrogen bond between alanine 128 and valine 131, resulting in an altered shape at the end of the active site cleft. Changes at position 130 are observed three times within the *bla*$_{\text{OXA-66}}$ gene group, with the proline residue being replaced by a leucine, serine or a glutamine residue (see appendix A), indicating that there is selection for the loss of proline at this site.

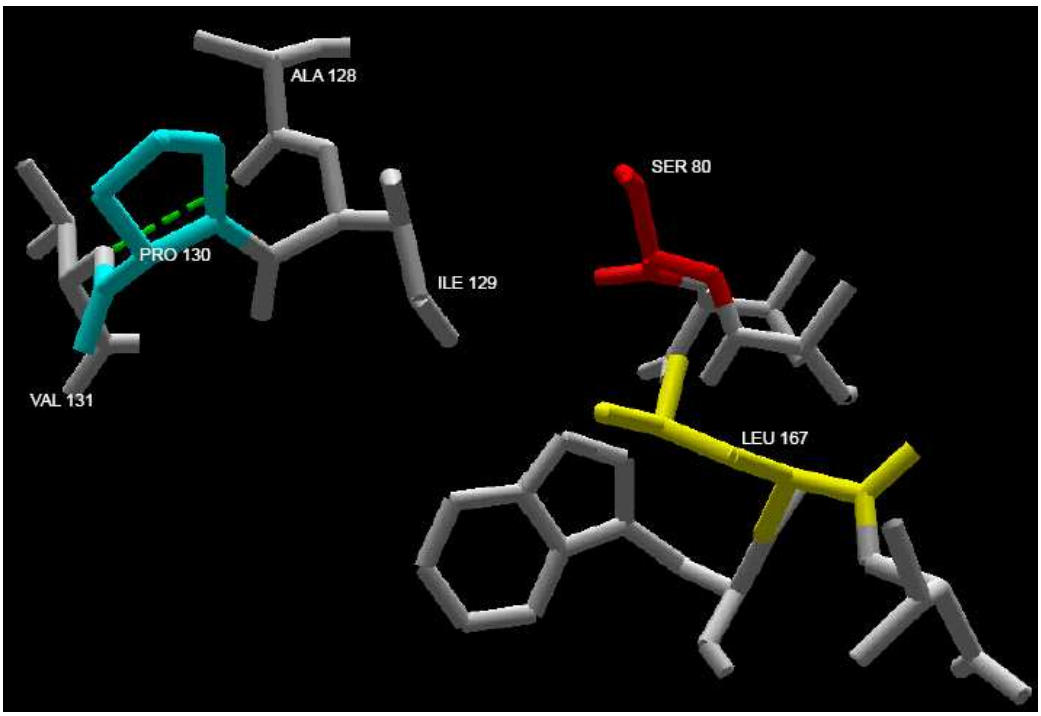

Figure 31: OXA-66 structure showing the residues proline 130 (blue), the hydrogen bond between alanine 128 and valine 131 (green dashed line), and leucine 167 (yellow), in relation to the active site serine 80 (red). Structure was visualised in DeepView.

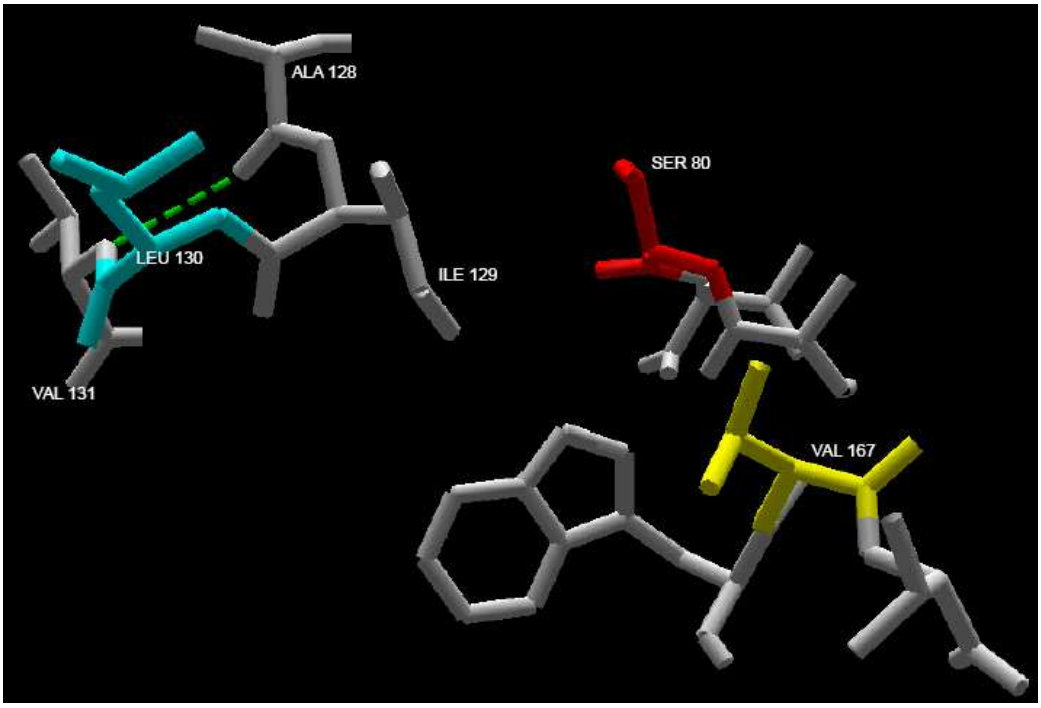

Figure 32: OXA-115 structure showing the residues leucine 130 (blue), the hydrogen bond between alanine 128 and valine 131 (green dashed line), and valine 167 (yellow), in relation to the active site serine 80 (red). Structure was visualised in DeepView.

3.10.2.4 Position 167.

Another position within the active site cleft at which there is amino-acid variation is position 167. A view down the active site cleft shown in figure 33, from the opposite direction as that shown in figures 29 and 30, reveals that enzymes such as OXA-66 have a leucine residue at position 167 that sits at the base and end of the cleft. The leucine residue is 5.25 Å from the active site serine 80, and only 3.70 Å from the isoleucine residue at position 129, which forms the other side of the base of the cleft, as shown in figure 29. However, other enzymes such as OXA-82, potential more recently evolved SG1 enzymes, have a valine residue at position 167 in place of the leucine. As shown in figure 34, this alters the shape of the end of the active site cleft. The valine residue sits further away from both the active site serine, 7.28 Å distant, as well as from the isoleucine residue at position 129, now at a distance of 6.12 Å. The presence of a valine residue at position 167 in place of a leucine residue increases the amount of space between the active site serine 80 and the end of the cleft, and also changes the shape of the end of the cleft. Changes at this position have occurred independently at least twice in the *bla*$_{\text{OXA-66}}$ clade, and once in the *bla*$_{\text{OXA-69}}$ and *bla*$_{\text{OXA-71}}$ clades of the *bla*$_{\text{OXA-51-like}}$ phylogeny, indicating that there is a significant degree of selection for this change. In OXA-115, a valine at position 167 combines with the loss of proline at position 130, which is likely to significantly alter the shape of the cleft in this enzyme (figure 32).

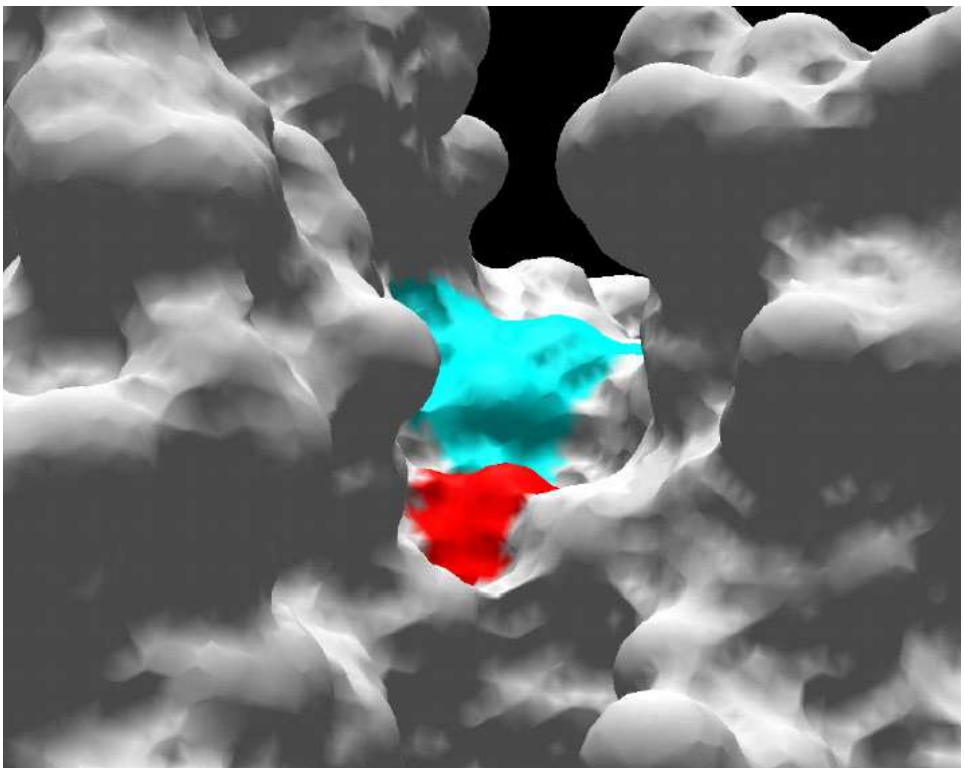

Figure 33: Molecular surface diagram showing view down the active site cleft of OXA-66 (grey) with the active site serine 80 (red) and a leucine at position 167 (blue) indicated. Structure was visualised in DeepView.

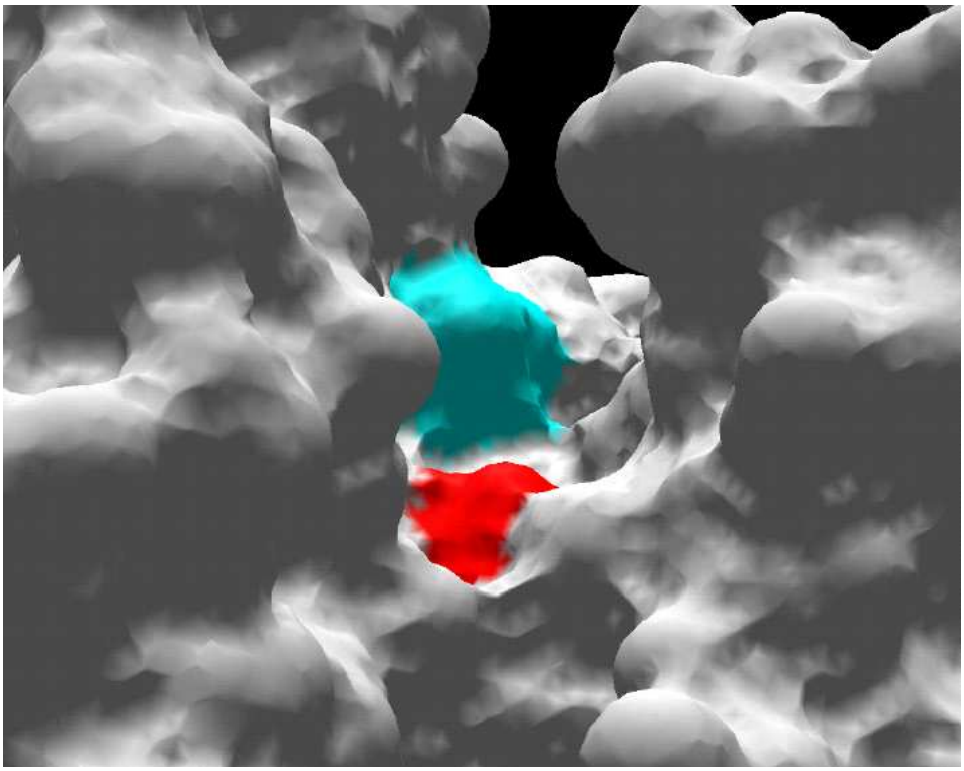

Figure 34: Molecular surface diagram showing view down the active site cleft of OXA-82 (grey) with the active site serine 80 (red) and a valine at position 167 (blue) indicated. Structure was visualised in DeepView.

3.10.2.5 Position 168.

Variation at position 168 is observed in two enzymes, OXA-84 and the enzyme with accession number EU255295. While the changes from a valine to an alanine or methionine respectively do not appear to cause any major structural changes within the models constructed, the proximity of this residue to the important position 167, and the fact that the changes at position 168 appear to have occurred independently within these two enzymes suggests that they may have an effect on the end of the active site cleft to which they are adjacent.

3.10.2.6 Position 225.

The final position that was investigated was 225. Residues at position 225 are on the surface of the enzyme and close to the entrance to the active site cleft. Some isolates such as OXA-66 have an asparagine residue at this position (figure 35). The structural models show this residue to form a hydrogen bond 3.17 Å long with the adjacent proline 226. Other enzymes such as OXA-71 have a aspartate residue at position 225, which while being slightly closer to the proline at position 226 (2.93 Å away) is twisted round, and a hydrogen bond is not formed (figure 36). The loss of a hydrogen bond at this position may allow more flexibility in these residues that are close to the active site cleft entrance, which may have implications for the conformation of the cleft. However, no structural changes were evident, which may be due to the limitations of the structural models based on the OXA-40 backbone.

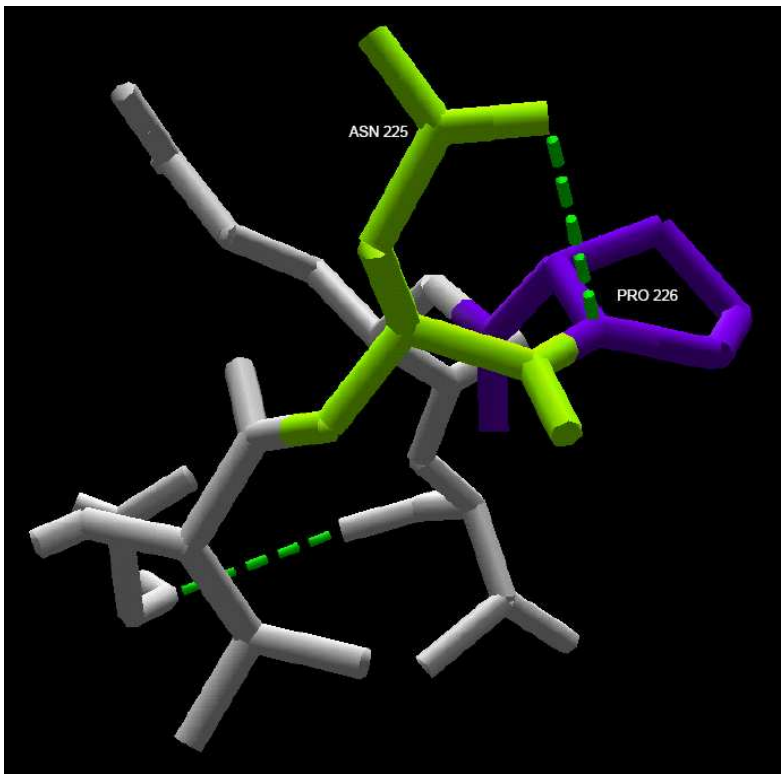

Figure 35: OXA-66 structure (grey) showing the asparagine at position 225 (green) hydrogen-bonding with the adjacent proline 226 (purple). Structure was visualised in DeepView.

Figure 36: OXA-71 structure (grey) showing the aspartate at position 225 (green) no longer hydrogen-bonding with the adjacent proline 226 (purple). Structure was visualised in DeepView.

4. Discussion.

The data presented in this thesis were obtained in order to answer questions about the nature of the *bla*$_{\text{OXA-51-like}}$ genes in *A. baumannii*. Within the literature, study of the *bla*$_{\text{OXA-51-like}}$ genes is very sparse, and combined with the relatively recent development of the sequence grouping scheme (Turton *et al*., 2007), this results in there being very few other data available with which the work presented here can be put into context. In fact, there is very few data available from large studies of chromosomally-located β-lactamases in any species, with the possible exception of the AmpC enzymes in *Citrobacter freundii* (Barlow and Hall, 2002). It is for these reasons that where there is discussion of other data here, the inability to make direct comparisons limits the conclusions that can be drawn from them.

4.1 Sequence grouping, insertion sequences and OXA-type β-lactamases.

The initial focus of this study was a phenotypic and genotypic analysis of a group of 64 *A. baumannii* isolates in order to compare the OXA-type β-lactamase gene and insertion sequence content of these isolates with their susceptibility to the carbapenems and the relatedness of the isolates. In the first instance the properties of the isolates were examined in relation to their categorisation by the sequencing grouping technique of Turton *et al* (2007), performed by Dr. Towner.

Within the isolates examined there was clearly a high representation of SG1 and SG2 isolates, which together accounted for 72% of all isolates. The isolates largely originate from Europe, and it may be that this biases the data set towards SG1 and SG2, as the lineages of European clones 2 and 1 (EC2 and EC1) which belong to

SG1 and SG2 respectively are known to be widespread in Europe, and to be responsible for many of the outbreaks of infection in this region (Towner *et al*., 2008). However, globally, reports of isolates related to EC2 (SG1) are widespread. In particular, reports from East Asia often find isolates related to EC2 to account for almost all *A. baumannii* that are identified (Kim *et al*., 2008, Cho *et al*., 2009). Analysis of isolates in our laboratory from Saudi Arabia and Pakistan has also identified 55% and 47% of isolates respectively to possess $bla_{\text{OXA-51-like}}$ genes that are associated with EC2 (Alsultan *et al*., 2009, and unpublished data). The reasons for the apparent global success of isolates belonging to the EC2 lineage, and the success of isolates belonging to EC1 in Europe, particularly countries in Eastern Europe, are unknown. It has been suggested that the reason for the success of *A. baumannii* as a species is due to the presence of the insertion sequence IS*Aba1* that is able to provide a promoter to increase gene expression levels as well as mobilise genes (Mugnier *et al*., 2009). It is interesting to note that IS*Aba1* is highly represented in the isolates belonging to SG1 and SG2 in this study, and this may be a contributing factor to their success. In contrast, isolates belonging to SG3 did not carry an IS*Aba1* element, and isolates from this sequence group are generally only identified in large numbers in studies examining outbreaks on the Iberian peninsula (van Dessel *et al*., 2004). It is possible that the lack of an IS*Aba1* element in SG3 isolates has prevented them from establishing themselves over a more widespread geographical area, as they lack the genetic flexibility provided by the insertion sequence. While the other sequence groups identified in this study contain too few representatives to support any reliable statements with regards to the prevalence of IS*Aba1* within them, it is interesting to note that IS*Aba1* is not widely found in the other sequence groups, with the

exception of SG4, which is associated with the *bla*$_{\text{OXA-51}}$ gene. Isolates encoding a *bla*$_{\text{OXA-51}}$ gene are reportedly widespread in South America, and it may be that SG4 represents another successful lineage similar to the SG1 and SG2 lineages in this geographic area (Merkier and Centron, 2006).

Within SG1 and SG2, the majority of *bla*$_{\text{OXA-51-like}}$ genes were found to be *bla*$_{\text{OXA-66}}$ and *bla*$_{\text{OXA-69}}$ respectively, with the remainder comprising genes very closely related to these. The reasons for these differences in frequency of *bla*$_{\text{OXA-51-like}}$ genes are unknown, but there are some interesting points to note. Within both SG1 and SG2, only isolates encoding *bla*$_{\text{OXA-66}}$ or *bla*$_{\text{OXA-69}}$ were also found to possess an acquired OXA-type β-lactamase gene of the *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-40-like}}$ or *bla*$_{\text{OXA-58-like}}$ families. Conversely, only isolates with *bla*$_{\text{OXA-51-like}}$ genes that were closely related to the *bla*$_{\text{OXA-66}}$ and *bla*$_{\text{OXA-69}}$ genes, rather than these genes themselves, were found to contain an IS*Aba1* element upstream of the *bla*$_{\text{OXA-51-like}}$ gene. This trend can be seen in data from studies such as that by Turton *et al* (2006a). In this study, of the nine isolates that had their *bla*$_{\text{OXA-51-like}}$ gene sequenced, the four isolates that carried a *bla*$_{\text{OXA-66-like}}$ gene all carried an IS*Aba1* element upstream of the gene, while the remaining five isolates that possessed *bla*$_{\text{OXA-66}}$ itself, or *bla*$_{\text{OXA-69}}$, were negative for IS*Aba1* upstream of the gene. As all but 4 of the 28 resistant or intermediately resistant isolates possessed either an acquired OXA-type β-lactamase or IS*Aba1* upstream of their *bla*$_{\text{OXA-51-like}}$ gene, it suggests that these two mechanisms represent two main paths that isolates within sequence groups can initially take in order to increase their resistance to the carbapenems, and that subsequently isolates may contain both, as seen in the two SG4 isolates A483 and A374.

Analysis of the OXA-51-like functional map demonstrates that while there is a reasonable degree of variation within the enzyme family as a whole, enzymes from isolates belonging to the same sequence group are typically only one or two amino-acids different to one another. Within the three main enzyme clusters surrounding OXA-66, OXA-69 and OXA-98, there is a trend for the most recently identified enzymes to be found at the edges of these clusters, suggesting that they may have evolved more recently. Additionally, the same amino-acid variations resulting in the more recently identified enzymes occur multiple times within and between the enzymes clusters, suggesting that these enzymes have come under a common selective pressure. It could be speculated that much of the amino-acid variation seen within the centre of the map is ancient and the result of chance mutations, whereas the more recently identified enzymes have arisen due to a common selective pressure such as antibiotic use. This would also explain why in this study the genes for 'founder' enzymes OXA-66 and OXA-69 were identified most frequently, as these enzymes would be older and so more widespread than the more recently evolved OXA-66-like and OXA-69-like enzymes, which have not had sufficient time to fix themselves in the *A. baumannii* population.

While the sequence grouping technique appears to be successful in classifying isolates, there are limitations to it that must be borne in mind. As the technique works through scoring isolates on whether or not there is a visible PCR amplification product within six separate multiplex PCRs it requires that a negative PCR result is interpreted as the absence of a particular allele. While generally this assumption is universally applied to PCR results, it has the potential to lead to the misclassification

of isolates into the wrong sequence group. In the present study, it is possible that this has happened with isolates A376 and A187. Isolate A376 is classified as belonging to SG5, while A187 belongs to SG9. The difference between these two sequence groups is within the *bla*$_{\text{OXA-51-like}}$ gene in the group 2 multiplex PCR. However, sequencing of the *bla*$_{\text{OXA-51-like}}$ gene from these two isolates identified a *bla*$_{\text{OXA-68}}$ sequence in both instances, suggesting that the isolates should both give the same result in any PCR that amplifies any region of this gene. In this case, it is likely that a false-negative result has caused these two isolates to be assigned to different sequence groups when they are in fact members of the same sequence group.

The current sequence grouping technique correlates well with specific *bla*$_{\text{OXA-51-like}}$ gene carriage, and the representative isolates of European clones 1, 2 and 3 (EC1, EC2 and EC3) are separated into SG2, SG1 and SG3 respectively. Due to this, throughout this study it is assumed, for example, that for all intents and purposes the terms EC1 and SG2, or the carriage of a *bla*$_{\text{OXA-69-like}}$ gene are effectively interchangeable. The same is true for EC2, SG1 and *bla*$_{\text{OXA-66-like}}$ gene carriage, and EC3, SG3 and *bla*$_{\text{OXA-71}}$ gene carriage. However, it should be noted that there has not been a study that assess in detail whether this is indeed the case. Such a study could be of great use, as at present it requires the use of several complementary typing techniques that are not routinely available in reference laboratories to determine whether isolates belong to one of the European clonal lineages. If it is found that identification of the *bla*$_{\text{OXA-51-like}}$ gene of an isolate is a proxy for these typing techniques, it would represent a much more simple, rapid and affordable method for determining the lineage that an isolate belongs to.

4.2 Carbapenem susceptibility.

Of the 64 isolates included in this study, 28 (44%) were resistant or of intermediate resistance to one or both of imipenem and meropenem, and all of these isolates belonged to SG1, SG2, SG3 or SG4. Generally imipenem was a more effective drug than meropenem, with MICs for isolates often being one to two-fold lower than those for meropenem. The reason for this difference is unknown. It may be that mechanisms such as the multidrug efflux pumps of the RND type which are found in *A. baumannii*, such as the AdeABC pump, that contribute to the resistance phenotype observed, have a better affinity for meropenem as a substrate than for imipenem.

A lot of attention has been focussed in the literature on the acquired OXA-type β-lactamases of *A. baumannii* and their contribution to carbapenem resistance in this species. Less attention has been focussed on the contribution of the $bla_{\text{OXA-51-like}}$ genes to carbapenem resistance, and how the over-expression of the $bla_{\text{OXA-51-like}}$ gene caused by the insertion of an IS*Aba1* element upstream of it compares with the resistance conferred by the acquired enzymes. In this study, a comparison of the imipenem and meropenem MICs for isolates with an acquired OXA-type β-lactamase only, with those with IS*Aba1* upstream of their $bla_{\text{OXA-51-like}}$ gene only, resulted in the rejection of the null hypothesis that the MICs for these two groups are different. This is an important finding, as it indicates that within the isolates in this study, the presence of an IS*Aba1* element upstream of the $bla_{\text{OXA-51-like}}$ gene has the same implications for carbapenem resistance as the presence of an acquired OXA-type β-lactamase. This is of great concern, as all *A. baumannii* carry a $bla_{\text{OXA-51-like}}$ gene, and within the most prevalent sequence groups the IS*Aba1* element is found at

a high frequency. If IS*Aba1* is able to mobilise itself to insert upstream of the *bla*$_{\text{OXA-51-like}}$ gene in most isolates, then this presents the worrying scenario whereby the majority of *A. baumannii* isolates could have MICs of the carbapenems at the same level as that which they would demonstrate if they possessed an acquired OXA-type β-lactamase.

An investigation of the effect of low-level expression of the *bla*$_{\text{OXA-51-like}}$ genes on an isolate's survivability to meropenem challenge compared with that seen in other *Acinetobacter* species through the measurement of dose response curves demonstrated that in the isolates examined without an IS*Aba1* element inserted upstream of the *bla*$_{\text{OXA-51-like}}$ gene there is no difference between the response of *A. baumannii* to meropenem challenge compared with other *Acinetobacter* species. A similar effect can be seen with regard to the *bla*$_{\text{OXA-23-like}}$ genes (Poirel *et al*., 2008). The *bla*$_{\text{OXA-23-like}}$ gene family is believed to have originated in the species *Acinetobacter radioresistens*. However, in this species the *bla*$_{\text{OXA-23-like}}$ genes are not associated with IS*Aba1* and isolates encoding the genes are fully susceptible to the carbapenems, suggesting that it is only when there is an association with IS*Aba1* and the resulting increased expression levels of the gene due to this that the *bla*$_{\text{OXA-23-like}}$ genes confer carbapenem resistance. Similarly, in this study it was only in isolate A343, which has an IS*Aba1* element upstream of the *bla*$_{\text{OXA-51-like}}$ gene, that the survivability to meropenem was much greater than that for the other *A. baumannii* isolates as well as the other *Acinetobacter* species tested. It is possible that it is through increased expression of the *bla*$_{\text{OXA-51-like}}$ gene in this isolate mediated by the IS*Aba1* sequence that is the cause for the increase in resistance level. However it

should be noted that the *bla*$_{\text{OXA-51-like}}$ genes that the isolates used for the dose response study carry are different, and it may be that the OXA-109 enzyme produced by isolate A343 has an increased ability to hydrolyse meropenem over the OXA-51-like enzymes produced by the other isolates, and that it is this difference that is responsible for the variation in resistance. Studies in which isogenic isolates either possessing IS*Aba1* upstream of the *bla*$_{\text{OXA-51-like}}$ gene or not, along with isogenic isolates carrying different *bla*$_{\text{OXA-51-like}}$ genes would allow these confounding factors to be separated.

Significantly, the MIC of meropenem to A343 suggests the isolate is of intermediate susceptibility, or fully susceptible depending on the criteria utilised, yet the isolate is able to survive meropenem concentrations at which isolates would be considered resistant while maintaining a high level of viability. This is an important point, as it means that data for isolates that are currently returned by hospital reference laboratories as being susceptible may be misleading, because if the isolate has an IS*Aba1* element upstream of its *bla*$_{\text{OXA-51-like}}$ gene it may be able to survive much higher concentrations of a carbapenem than the MIC suggests.

4.3 Pulsed-field gel electrophoresis.

Analysis of the PFGE profiles of the 64 isolates in this study was not able to group isolates in concordance with the *bla*$_{\text{OXA-51-like}}$ gene that they possess, unlike the sequence grouping technique. Not only were isolates with the same *bla*$_{\text{OXA-51-like}}$ gene not grouped together by PFGE, but isolates with different *bla*$_{\text{OXA-51-like}}$ genes from different sequence groups were clustered more closely to one another than they were

to the other isolates from their sequence group. The technique of PFGE can be very useful for examining the relatedness of isolates collected over a reasonably long period of time for which there is little lateral gene transfer and low mutation rates, as changes in the restriction sites in such isolates will accumulate slowly. The technique can also be applied to isolates within which the rate of lateral gene transfer and mutation may be higher, as long as the isolates represent a much shorter timeframe, within which there has not been sufficient time for a large number of changes at restriction sites to occur. The 64 isolates examined in this study were collected over a period of 25 years between 1982 and 2006. The failure of PFGE to group isolates together as might be expected from the sequence grouping results suggests that either the degree of lateral gene transfer, the mutation rates, or both, are high enough that PFGE is unable to cluster isolates in a manner that is representative of their relatedness. Alternatively, it is possible that the $bla_{\text{OXA-51-like}}$, *csuE* and *ompA* genes that are used for the sequence grouping scheme, which are all implicated in antimicrobial resistance or virulence in *A. baumannii*, may have been subject to horizontal gene transfer. As such the PFGE profiles would present a more representative picture of the relatedness of the whole genomes of the isolates examined. In order to determine whether the sequence grouping technique or PFGE was more representative of the relatedness of the isolates, a subset of the isolates were typed by MLST.

4.4 Multi-locus sequence typing.

MLST is a technique which is commonly used in bacteria to determine the phylogenetic relationships of isolates. It is based upon sequencing regions of a

number of genes that are chosen based upon being relatively conserved within the species under examination. Often, genes for essential cellular functions, so-called 'housekeeping' genes, are used. This makes MLST an excellent method for analysing longer-term evolution within and even between species, but will not be able to distinguish between recently diverged isolates. There are many different schemes for many different bacterial species, each with their own selection of 'housekeeping' genes that are amplified. For *A. baumannii* there is an MLST scheme published by Bartual *et al* (2005), as well as an online scheme (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html). In this study the scheme of Bartual *et al* was used, which utilises the sequences from seven genes.

Analysis of the allelic profiles of the representative subset of 44 isolates by eBURST grouped the isolates into four clonal complexes, with the remainder of the isolates represented as singletons. All of the sequence types (STs) that are linked with ST34, which together form the largest clonal complex, are from isolates that belong to SG1. The STs associated with ST33, the second largest clonal complex, are all from isolates belonging to SG2. The remaining two clonal complexes that consist of two STs each (ST50 and ST48, and ST35 and ST38) belong to isolates assigned to SG4 and SG3 respectively. Combined with the index of association value calculated that is significantly greater than zero, this demonstrates that SG1, SG2, SG3 and SG4 are indeed discrete lineages that are quite different to one another. However, when the actual nucleotide sequences for the alleles were used to construct a more accurate phylogeny, the resulting tree had split the SG2 isolates into separate clades. Following a more detailed analysis, two of the alleles, *gyrB* and *gpi*, showed

significant evidence for recombination. As the MLST method rests upon the assumption that in the majority of isolates for the majority of the time the alleles under investigation are stable, then alleles that appear to be undergoing a significant amount of horizontal gene transfer are unsuitable. As such, the *gyrB* and *gpi* loci should not be considered suitable for use in an MLST scheme. The only study to have published an in-depth analysis of results using the MLST scheme of Bartual *et al* (2005) appears to have the same problems as were encountered here, with the trees for *gyrB* and *gpi* being non-concordant with those for the other loci (Park *et al*., 2009). Interestingly, there was a reasonable degree of discordance with the tree for the *gdhB* alleles and the trees for the other six alleles as well, which was not observed in our data. Unfortunately neither this study nor the other published studies that have utilised the Bartual *et al* MLST scheme presented sequence grouping or $bla_{\text{OXA-51-like}}$ allele data that could be used to compare with the data generated here (Bartual *et al*., 2005, Wisplinghoff *et al*., 2008, Park *et al*., 2009).

Analysis of the data using the remaining five loci produced a phylogeny that is far more concordant with sequence grouping, $bla_{\text{OXA-51-like}}$ gene content, and the population structure suggested by the eBURST analysis. However, there are a few notable exceptions, with two SG1 isolates separated from each other and from the main SG1 clade, one SG2 isolate separated from the main SG2 clade, and one SG4 isolate separated from the other two SG4 isolates. In all of these cases there is a common feature, in that these isolates differed from the other isolates in their sequence groups at the *gdhB* and *cpn60* loci. While some of these isolates differed at more than just these two loci, this common feature suggests that these two loci are

more variable than the *gltA*, *rpoD* and *recA* loci. However, in the case of the SG4 isolate A483, which differed from the other two SG4 isolates at all five MLST loci, it is possible that it is the *bla*$_{\text{OXA-51-like}}$ gene that has been horizontally transferred, though due to the results of the eBURST analysis indicating that the sequence types of the SG4 isolates are related it is perhaps more likely that isolate A483 has a much higher mutation rate than the other SG4 isolates and as such has one or two nucleotide changes in each of its 'housekeeping' genes. While the isolates share the same sequence group, this does not necessarily mean that the *ompA* and *csuE* genes in these three isolates are identical or very similar, as they may vary considerably outside of the regions in which the multiplex primers bind. Fully sequencing the *ompA* and *csuE* genes in the three SG4 isolates would indicate whether it was more likely that the *bla*$_{\text{OXA-51-like}}$ gene had been horizontally transferred, whether there has been a significant degree of horizontal gene transfer involving many loci, or there is a higher rate of mutation in isolate A483. While it is generally assumed that the *bla*$_{\text{OXA-51-like}}$ genes are immobile and maintained on the *A. baumannii* chromosome, there has been a brief mention in the literature of potential horizontal transfer of the genes (Pournaras *et al*., 2006), and very recently the sequence of a *bla*$_{\text{OXA-51-like}}$ gene from a genospecies 13TU isolate in association with an upstream IS*Aba1* element has been deposited in GenBank (accession number EU670845.2), indicating that there is potential for these genes to be horizontally transferred.

Currently the evidence for horizontal transfer of the *bla*$_{\text{OXA-51-like}}$ genes is sparse. As the genes can be associated with an IS*Aba1* element located upstream, which has been implicated in the mobility of the *bla*$_{\text{OXA-23-like}}$ genes (Poirel *et al*, 2008; Corvec

et al, 2007), it is conceivable that the *bla*$_{\text{OXA-51-like}}$ genes could also be mobilised in this manner. Whether the reports of mobilised *bla*$_{\text{OXA-51-like}}$ genes represent the start of the 'escape' of these enzymes from the *A. baumannii* chromosome, or whether they represent infrequent spontaneous recombination events with plasmids already carrying an IS*Aba1* sequence remains to be seen. Should the *bla*$_{\text{OXA-51-like}}$ genes confer an advantage to the isolates that acquire them in terms of increased antibiotic resistance, then it is likely that these genes will join the ever-increasing group of mobile antibiotic resistance determinants. Interestingly, the only *bla*$_{\text{OXA-51-like}}$ gene described to have been horizontally transferred, *bla*$_{\text{OXA-138}}$ found in an *Acinetobacter* species 13TU isolate, is the gene at the very tip of the *bla*$_{\text{OXA-66}}$ clade in the *bla*$_{\text{OXA-51-like}}$ gene tree (figure 25), potentially representing one of the most recently evolved *bla*$_{\text{OXA-51-like}}$ genes. It contains the leucine to valine substitution at position 167, presenting the possibility that this gene has recently evolved with altered kinetic characteristics, which may have resulted in the mobilised gene being selected for as it conferred an advantage to the recipient isolate.

It should be noted that as the sequence grouping was performed by Dr. Towner in Nottingham, and the *bla*$_{\text{OXA-51-like}}$ gene sequencing and MLST was conducted in Edinburgh, it is possible that isolate mix-up, resulting in the two laboratories working on different isolates but believing them to be the same one, could explain discrepancies between the MLST and SG results. However, within the isolates where such discrepancy was noted in this study, the SG and *bla*$_{\text{OXA-51-like}}$ identification were concordant, while the MLST was not. As the MLST and *bla*$_{\text{OXA-51-like}}$ sequencing

were both done in Edinburgh, it is highly unlikely that an isolate mix-up between the two laboratories explains the discrepancy between MLST and SG designation.

It has been suggested that the *bla*$_{\text{OXA-51-like}}$ genes of *A. baumannii* are themselves 'housekeeping' genes akin to those that would be used in an MLST scheme (Whitman *et al*., 2008). This study demonstrates that the degree of diversity within the *bla*$_{\text{OXA-51-like}}$ alleles as measured by the $\pi N/\pi S$ ratios is far greater than that seen within the seven 'housekeeping' genes from the MLST scheme, with the ratio a minimum of 3.4-fold higher. When compared with a range of other β-lactamase genes that are widely accepted as arising through antibiotic selection, the $\pi N/\pi S$ ratio for the *bla*$_{\text{OXA-51-like}}$ genes sits within the range of 0.1664 - 0.4357 that is calculated for the ratios for these genes, suggesting that the *bla*$_{\text{OXA-51-like}}$ genes are under a similar level of positive selection as these other β-lactamase genes. However, all of the loci tested gave ratios that were less than one. The likely explanation for this is that the ratios were calculated across the whole allele sequence. The structures of the β-lactamase enzymes are highly conserved, with enzymes from classes A, B, C and D demonstrating a remarkable similarity in tertiary structure. As was shown in figure 27 in the results section, using a sliding window across the *bla*$_{\text{OXA-51-like}}$ allele sequences to calculate $\pi N/\pi S$ ratios demonstrates that the majority of the enzyme sequence is under purifying selection, with only a few 'hotspots' where the $\pi N/\pi S$ ratio was greater than one. The effect of this is that while the majority of the sequence is under purifying selection such that the general structure of the β-lactamase is maintained, any non-synonymous nucleotide changes that occur in or around the active site region due to positive selection are in the minority compared to

the synonymous changes across the rest of the sequence. As mentioned above, when ratios were calculated for different regions across the enzyme sequence, it was demonstrated that some regions of the enzyme are under a higher level of positive selection than others. Ideally, the most precise analysis to detect which sites were under a greater degree of positive selection than others would be obtained by measuring the $\pi N/\pi S$ ratios across smaller regions between enzymes. However, with the β-lactamase enzymes, in many cases this will not be possible, as the only difference between two enzymes is one amino-acid substitution as a result of a single nucleotide substitution. In these cases a ratio could not be calculated as the value for πS would be zero. It should also be noted that the other β-lactamase families that were used for comparison with the OXA-51-like enzymes were comprised entirely of alleles that are believed to have arisen due to antibiotic selection. For example, with the *bla*$_{SHV}$ genes, a range of ancestral sequences from *Klebsiella pneumoniae* that were in existence before the advent of antibiotics were not included. This inherently biases the $\pi N/\pi S$ ratio towards a higher value. As was discussed previously, it is possible that a large number of the OXA-51-like enzymes are not enzymes that have recently evolved in response to antibiotic use, but rather are much older enzymes. If this is the case, then a $\pi N/\pi S$ ratio for these enzymes that is calculated using the sequences for all of the group members will inevitable be lower than one calculated using enzymes that are thought to have arisen through antibiotic selection alone. Unfortunately there is not enough information available at present to determine which of the OXA-51-like enzymes are likely to have arisen due to recent antibiotic selection in the same manner as the other β-lactamase families, but it is likely that if

such a like-for-like comparison of πN/πS could be made then the ratios for the *bla*$_{\text{OXA-51-like}}$ genes would be even higher than those calculated in this study.

4.5 *A. baumannii* mutability.

Two experiments were undertaken in this study to examine the degree of genetic variability in *A. baumannii* over time. While the mutation study was able to obtain isolates that were able to grow at repeatedly higher concentrations of meropenem, no nucleotide changes were seen in the *bla*$_{\text{OXA-51-like}}$ genes. This is perhaps not surprising, as the bacteria are able to rapidly adapt to an increasing concentration of antibiotic by regulating the expression of efflux systems such as the AdeABC pump, and outer membrane porins. Changes in gene regulation are able to occur far more rapidly than mutations, as mutations are random events that occur at a relatively low frequency.

Additionally, IS*Aba1* elements were not found to have inserted upstream of the *bla*$_{\text{OXA-51-like}}$ genes. However, this has been observed in a clinical isolate from France in which the isolate, following treatment of the patient with imipenem, gained an IS*Aba1* element upstream of its *bla*$_{\text{OXA-51-like}}$ gene that was not present prior to treatment (Figueiredo *et al.*, 2009). This process does not seem to have been observed frequently, which may be for a number of reasons. Firstly, it may be that it is only within certain isolates that IS*Aba1* elements are able to move frequently. Certainly it has been shown that different *A. baumannii* lineages are not equal in the frequency that they carry IS*Aba1*, and so it may be the case that there is additional variation within the species in the degree to which isolates facilitate the mobilisation

of the element once it is present. Secondly, if the IS*Aba1* elements are not stable, then the removal of the antibiotic pressure may result in the element excising from the upstream region of the *bla*$_{\text{OXA-51-like}}$ gene. In our laboratory we have observed that isolates initially reported as encoding IS*Aba1* upstream of their *bla*$_{\text{OXA-51-like}}$ genes can later be found to be negative, following a period of time in which the isolates were stored at -80°C. Additionally, during a sequencing project of *A. baumannii* genomes problems were encountered due to IS*Aba1* elements moving between sequencing runs (Mark Adams, personal communication). It may be that in many cases an IS*Aba1* element is not identified upstream of *bla*$_{\text{OXA-51-like}}$ genes as in the period between retrieving the isolate from the patient and a study being conducted the element has moved.

In addition to increased expression of the *bla*$_{\text{OXA-51-like}}$ gene mediated by an IS*Aba1* sequence, it is possible that carbapenem MICs for isolates could be increased through duplication of the *bla*$_{\text{OXA-51-like}}$ gene. Such a phenomenon has been observed with the *bla*$_{\text{OXA-58}}$ gene, where duplication of the gene mediated by IS26 elements resulted in isolates with one, two or three copies of the gene. The increase in copy number was seen to be associated with a step-wise increase in the MICs of meropenem and imipenem (Bertini *et al*, 2007). In the isolates studied here, while the presence of IS*Aba1* upstream of the *bla*$_{\text{OXA-51-like}}$ genes was analysed, the presence of other insertion sequences was not, and it is possible that the *bla*$_{\text{OXA-51-like}}$ genes in some of the isolates were being duplicated by an unknown mechanism. Sequencing the surrounding genetic environment of the *bla*$_{\text{OXA-51-like}}$ genes in the isolates with raised

carbapenem MICs combined with southern analysis of restricted DNA would determine if this were the case.

In the second experiment where an *A. baumannii* isolate was grown continuously for 100 days under both nutrient-rich conditions and sub-inhibitory concentrations of imipenem, there were again no differences observed in the sequence of the $bla_{\text{OXA-51-like}}$ gene, and the IS*Aba1* element did not insert upstream of the $bla_{\text{OXA-51-like}}$ gene. Additionally, the sequences of the MLST alleles were identical in both the parent and the 100-day-old isolates. This was as expected following the results of the previous mutation study, and the fact that the 'housekeeping' genes are, due to their nature, relatively highly conserved. However, analysis of the PFGE patterns of the isolates showed that there was a small difference in the banding pattern of the isolate that had been grown under sub-inhibitory concentrations of imipenem compared to the parent isolate. After 100 days of growth at a sub-inhibitory concentration of imipenem the isolate demonstrated only 88% relatedness with its parent isolate. The reason for the alterations in the restriction sites and therefore the banding patterns is unknown, but it is interesting that the differences were only observed in the isolate that was under selective pressure and not the isolate grown in non-selective nutrient-rich conditions. This result is consistent with the PFGE data obtained for the 66 isolates in this study where isolates that are shown to belong to the same lineage by sequence grouping and MLST techniques were completely unrelated by PFGE. In the hospital, bacteria are exposed to much higher concentrations of antibiotic than were used here, as well as a range of other selective pressures such as exposure to biocides. The results of the growth study indicate that over a relatively short period of time under little selective

pressure the PFGE profile of an isolate can vary, and that for *A. baumannii* care should be taken in interpreting PFGE data if isolates have been obtained over a period of more than a few months, particularly if the isolates originated from environments in which there is considerable selective pressure.

4.6 Sequence analysis.

Analysis of the phylogeny of *A. baumannii* as a species and of the $bla_{\text{OXA-51-like}}$ genes provides substantial evidence that within the species there are discrete lineages that have been evolving independently of one another for a significant period of time, and that the $bla_{\text{OXA-51-like}}$ genes associated with these lineages have been co-evolving within them.

The species tree for *A. baumannii* is intriguing as the different clades extend varying distances from the root. Two explanations for this topology are that the different branch lengths represent differences in time, with the isolates closest to the root being the oldest and those furthest away the youngest, or that they represent differences in mutation rate, with those isolates closest to the root evolving more slowly and those furthest from the root evolving more rapidly. As the isolates used in this study, and all of the sequenced isolates that were downloaded from PubMed, were all isolated relatively recently, particularly in evolutionary terms, then the differences in branch lengths that are observed are likely to represent differences in the rates of evolution of different clades. This is an interesting finding as the identification of different lineages within a bacterial species evolving at markedly

different rates is unusual and suggests a significant degree of heterogeneity within *A. baumannii* as a species.

The conclusions that can be drawn from the topology of the *A. baumannii* species tree are reinforced when the phylogeny of the $bla_{\text{OXA-51-like}}$ genes is examined. As with the species tree, the sequences of the $bla_{\text{OXA-51-like}}$ genes have all been identified from isolates that are recent in origin, therefore the variations in branch length are likely as the result of varying rates of mutation. However, in the $bla_{\text{OXA-51-like}}$ tree the variation in the branch lengths of the different clades is even more pronounced than in the species tree. Importantly, there is a correlation in the lengths of the branches between the isolates and $bla_{\text{OXA-51-like}}$ genes of the three main sequence groups, with SG1 isolates and $bla_{\text{OXA-66-like}}$ genes on the longest branches of the three groups, SG3 isolates and $bla_{\text{OXA-71}}$ genes on the shortest branches, and SG2 isolates and $bla_{\text{OXA-69-like}}$ genes on branches of an intermediate length. Additionally the three groups of enzymes that are associated with the three main sequence groups are found in discrete clades that all diverge from the root, in the same manner as the clades for the isolates belonging to the different sequence groups. These observations support the suggestion that the different sequence groups have been evolving independently and at different rates. However, it should be noted that only four alleles were used to construct the *A. baumannii* species tree. While the overall topology of the species tree is unlikely to be substantially altered with the inclusion of a greater number of reliable 'housekeeping' genes, due to the concordance with the $bla_{\text{OXA-51-like}}$ tree and with the sequence grouping data, the relationships between some of the branches

belonging to isolates that are not from the main sequence groups may be subtly altered.

An interesting feature of the *bla*$_{\text{OXA-51-like}}$ tree is its ladder-like topology seen in some branches. Such topologies are usually seen in trees for rapidly evolving organisms such as the influenza A virus, where the topology is indicative of repeated selective sweeps of the population (Cobey and Koelle, 2008). Interestingly it is the branches that are thought to be evolving more quickly and that are associated with the major epidemic lineages of SG1 and SG2 that show the most pronounced ladder-like topology, though most of the branches do to some extent. This suggests that the *bla*$_{\text{OXA-51-like}}$ genes, and in particular those belonging to SG1 and SG2 isolates, have been periodically subjected to positive selection that has served to reduce the variation within the clades of this enzyme family. Due to the assumed intrinsic nature of the *bla*$_{\text{OXA-51-like}}$ genes to *A. baumannii*, it is likely that much of the positive selection resulting in the topology seen in the gene tree was acting upon the isolate genomes as a whole rather than upon the *bla*$_{\text{OXA-51-like}}$ genes themselves, and this corresponds with the fact that the basic topology of the *A. baumannii* species tree is concordant with the *bla*$_{\text{OXA-51-like}}$ tree.

Comparisons of the phylogenies for isolates from the three main sequence groups and the *bla*$_{\text{OXA-51-like}}$ genes associated with them demonstrate not only a correlation between the branch lengths of the sequence groups and their *bla*$_{\text{OXA-51-like}}$ genes, but in each case the relative evolutionary rate of the *bla*$_{\text{OXA-51-like}}$ genes is considerably higher than the rate for the isolates from the sequence group that contain them. This

suggests that the *bla*$_{\text{OXA-51-like}}$ genes are consistently evolving at a faster rate than the *A. baumannii* core genome, as represented by the 'housekeeping' genes. This is in agreement with the $\pi N/\pi S$ ratios calculated for the *bla*$_{\text{OXA-51-like}}$ genes and the MLST alleles. As the *bla*$_{\text{OXA-51-like}}$ genes are under less purifying selection than the 'housekeeping' genes they will have a faster rate of fixation of mutations generated by random genetic drift, and therefore greater variation. This results in their rate of evolution being closer to the mutation rate, while the rate of evolution of the 'housekeeping' genes is far slower than the mutation rate, as purifying selection removes the majority of deleterious mutations that arise.

Analysis of the *bla*$_{\text{OXA-51-like}}$ phylogeny confirms some of the observations made with regards to the OXA-51-like enzyme functional map. Firstly, as mentioned above the distinct clades within the *bla*$_{\text{OXA-51-like}}$ tree all diverge from the root, with the vast majority of enzymes being located at a distance from the root. This correlates with the splitting of the enzymes into cluster in the functional map, and a lack of enzymes in the central region. Additionally, the enzymes that are found at the edges of the three main clusters surrounding OXA-66, OXA-69 and OXA-98 in the functional map tend to be found at the extremities of their clades in the *bla*$_{\text{OXA-51-like}}$ tree. It is likely that the *bla*$_{\text{OXA-51-like}}$ genes that are found at the tips of their clades are the sequences that have evolved most recently. However, since the discovery of the existence of the *bla*$_{\text{OXA-51-like}}$ genes was only published in 2005 (Brown *et al*., 2005), combined with the fact that *A. baumannii* has only been recognised as an important pathogen for a few decades, means that almost all of the *bla*$_{\text{OXA-51-like}}$ sequences that are now known are from relatively recent isolates. Therefore there is no historical

timeline that can be followed for the evolution of these enzymes as there is for enzymes such as the TEM and SHV families. However, the *bla*$_{\text{OXA-51-like}}$ tree gives a well supported estimation of the evolutionary history of the genes, and is backed up by the frequency of identification of different *bla*$_{\text{OXA-51-like}}$ genes. For example, in the top-most clade of the tree, the genes that are positioned closer to the root of the tree have only been identified on one occasion, demonstrating that while they are still in existence, they are at a low frequency, as would be expected had these genes been the ones that were severely depleted in frequency during a selective sweep. On the other hand, the gene *bla*$_{\text{OXA-66}}$ is found towards the tip of the clade in a region of the tree where the ladder-like topology is no longer evident. The *bla*$_{\text{OXA-66}}$ gene is the most frequently identified *bla*$_{\text{OXA-51-like}}$ gene in *A. baumannii*, and it is likely that this gene is currently the most successful within this clade. The shape of the tree at this point, where the genes are all diverging from the same node as *bla*$_{\text{OXA-66}}$, suggests that these other genes have all arisen recently from *bla*$_{\text{OXA-66}}$. This is supported by the fact that these other genes have only been found in low numbers. If further selective sweeps occur in this clade as appear to have done previously, this will greatly reduce the frequency of most of the genes in this grouping, with the successful gene going on to found the next grouping of closely related enzymes, as *bla*$_{\text{OXA-66}}$ is currently doing. The same process appears to be happening in the *bla*$_{\text{OXA-69}}$ clade and the *bla*$_{\text{OXA-98}}$ clade, though is less evident in the other clades.

The structure of the *bla*$_{\text{OXA-51-like}}$ tree indicates, as suggested by the OXA functional map, that *bla*$_{\text{OXA-98}}$ and similar genes form a distinct clade. Unfortunately, within the 64 isolates included in this study only 3 possessed *bla*$_{\text{OXA-51-like}}$ genes that are found

in the *bla*$_{\text{OXA-98}}$ clade, and so an analysis of whether isolates that carry this group of genes form a major sequence group cannot be made. However, it should be noted that the three isolates that were identified with *bla*$_{\text{OXA-98-like}}$ genes in this study were all assigned to separate lesser sequence groups. The isolate that were used in the development of the sequence grouping scheme almost all carried a *bla*$_{\text{OXA-66-like}}$, *bla*$_{\text{OXA-69-like}}$, or *bla*$_{\text{OXA-71}}$ gene, and none carried a *bla*$_{\text{OXA-98-like}}$ gene. Therefore, it is perhaps unsurprising that the scheme is able to easily assign isolates encoding *bla*$_{\text{OXA-66-like}}$, *bla*$_{\text{OXA-69-like}}$ and *bla*$_{\text{OXA-71}}$ genes to SG1, SG2 and SG3 respectively, but when isolates encoding other *bla*$_{\text{OXA-51-like}}$ genes are encountered, the scheme does not group together isolates that might be expected to be of the same sequence group based upon their *bla*$_{\text{OXA-51-like}}$ genes. Further development of the sequence grouping scheme to include a wider range of isolates encoding a greater variety of *bla*$_{\text{OXA-51-like}}$ genes may result in the identification of further major sequence groups similar to SG1, SG2 and SG3.

The conclusion that the *bla*$_{\text{OXA-51-like}}$ genes have been evolving within *A. baumannii* for a significant period of time raises the question of why these genes have been maintained in this species. It has been known for some time that many bacteria possess a chromosomal class C β-lactamases, the expression of which is able to confer resistance to the cephalosporin β-lactams (Jacoby, 2009). The cephalosporins are derived from molecules that are produced naturally by a number of soil bacteria and fungi, and the class C beta-lactamases are likely to have evolved in response to the presence of these antibiotics in the environment (Brakhage *et al.*, 2005). *A. baumannii* is one of many species that possess a class C enzyme, which in the case of

A. baumannii are referred to as ADC enzymes (for *Acinetobacter*-derived cephalosporinase) (Hujer *et al*., 2005). In contrast to the class C enzymes, molecular class D enzymes such as the OXA-51-like family are much more efficient at hydrolysing the penicillins than the cephalosporins (Bush *et al*., 1995). Like the cephalosporins, the penicillins are also derived from naturally-produced molecules from soil bacteria, and similarly the evolution of penicillinases is thought to have occurred in response to this (Brakhage *et al*., 2005). Recent discoveries of chromosomally-located class D β-lactamases in many bacterial species (table 18) indicate that like the class C enzymes, the presence of a gene encoding a class D β-lactamase within certain bacterial classes is not an uncommon occurrence. In the evolutionary arms race that bacterial species are engaged in both with other bacteria and with other domains of life, bacterial species have evolved multiple antibiotic defences, and in the case of *A. baumannii* it is possible that the $bla_{\text{OXA-51-like}}$ genes evolved as a defence against penicillins while the bla_{ADC} genes evolved as a defence against cephalosporins.

Enzyme	Species	Class	Order
OXA-12	*Aeromonas jandaei*	Gamma-Proteobacteria	Aeromonadales
OXA-22	*Ralstonia pickettii*	Beta-Proteobacteria	Burkholderiales
OXA-23	*Acinetobacter radioresistens*	Gamma-Proteobacteria	Pseudomonadales
OXA-29	*Legionella gormanii*	Gamma-Proteobacteria	Legionellales
OXA-50	*Pseudomonas aeruginosa*	Gamma-Proteobacteria	Pseudomonadales
OXA-51	*Acinetobacter baumannii*	Gamma-Proteobacteria	Pseudomonadales
OXA-54	*Shewanella oneidensis*	Gamma-Proteobacteria	Alteromonadales
OXA-55	*Shewanella algae*	Gamma-Proteobacteria	Alteromonadales
OXA-60	*Ralstonia pickettii*	Beta-Proteobacteria	Burkholderiales
OXA-61	*Camylobacter jejuni*	Epsilon-Proteobacteria	Campylobacterales
OXA-62	*Pandoraea pnomenusa*	Beta-Proteobacteria	Burkholderiales
OXA-63	*Brachyspira pilosicoli*	Spirochaetes	Spirochaetales

Table 18: Selected chromosomally-located class D OXA-type β-lactamases. Data for OXA-12 from Rasmussen *et al* (1994), OXA-22 and OXA-60 from Girlich *et al* (2004b), for OXA-23 from Poirel *et al* (2008), for OXA-29 from Franceschini *et al* (2001), for OXA-50 from Girlich *et al* (2004a), for OXA-51 from Brown *et al* (2005), for OXA-54 from Poirel *et al* (2004), for OXA-55 from Heritier *et al* (2004), for OXA-61 from Alfredson and Korolik (2005), for OXA-62 from Schneider *et al* (2006), and for OXA-63 from Meziane-Cherif *et al* (2008).

4.7 OXA-51-like structural analysis.

Despite that fact that all of the *bla*$_{\text{OXA-51-like}}$ genes have been identified recently, it is likely that it is the genes that are found towards the tips of the various clades in the *bla*$_{\text{OXA-51-like}}$ phylogeny that have evolved most recently. Therefore, any evolution within the *bla*$_{\text{OXA-51-like}}$ family that has taken place due to carbapenem selective pressure is likely to be manifest in these tip-located genes. However, in order not to bias the identification of important structural changes by this assumption, an analysis of variance across all of the *bla*$_{\text{OXA-51-like}}$ genes was conducted.

The analysis of $\pi N/\pi S$ ratios and percentage amino-acid variation from a consensus sequence identified 12 amino-acid positions which would be examined in detail

using structural models. However, for seven of these positions no structural changes were observed. The reason for this may be due to the limitations of the method used to construct the structural models. As there is no crystal structure available for any of the OXA-51-like enzymes, the structure for the OXA-40 enzyme had to be used (Santillana *et al*., 2007). The OXA-51-like models were constructed by the sequences for these enzymes being super-imposed on the OXA-40 backbone. While this approach is useful in allowing the positions that the different amino-acids occupy to be visualised, it is not able to model major changes to the protein backbone that may be caused by the differences in the amino-acid sequences between OXA-40 and the OXA-51-like enzymes. It is likely that it is limitations in the structural models that have resulted in no structural changes being identified for some of the 12 amino-acid positions investigated. For example, the change at position 194 from a proline in enzymes such as OXA-51 to a glutamine in enzymes such as OXA-66 and OXA-69 is likely to have a significant structural impact, particularly considering its position in a loop that joins two central α helices. However, as the structural models are not able to model such major structural changes, no differences are seen between the structures of OXA-51, OXA-66 and OXA-69 due to changes at position 194. To enable the actual effects that these amino-acid variations have on the structures of the OXA-51-like enzymes requires a crystal structure to be determined for one of them that can then be used as a basis for more reliable models of the other enzymes to be determined.

Another reason that differences at some of the amino-acid positions investigated did not show any structural variation in the structural models is that these amino-acids

are not involved in actual structural changes in the enzyme itself, and vary for another reason that cannot be determined with the models available. For example, the β-lactamases OXA-2, OXA-10 and OXA-13 exist in the form of dimers when at high enough concentrations (Paetzel *et al*., 2000, Pernot *et al*., 2001, Maveyraud *et al*., 2002), such as might be found in the bacterial periplasmic matrix. There has also been a suggestion that OXA-69 may also exist in a dimerised form (Heritier *et al*., 2005a). If the OXA-51-like enzymes exist in the form of dimers when expressed at a high enough level, then there is the potential for amino-acid variation to arise on the surface of the proteins that results in variation in properties such as the binding affinity of the two monomers to one another. It is interesting to note that of the seven amino-acid positions that appeared to show no structural changes in the enzyme, six are positioned on the enzyme surface, with the seventh just below the surface of the enzyme. However, none of these amino-acid positions are located within the end of the β3 strand or the α9 helix, which are implicated in dimerisation in the other class D enzymes. It is possible that some of these amino-acid variations are responsible for changes in how the protein interacts with other molecules, rather than having a direct affect on the protein structure itself.

The final reason for why variation at some amino-acid positions appears to have no structural effect may be because the variation is due to stochastic effects. For example, an amino-acid change that occurred far back in the evolutionary history of the OXA-51-like enzymes simply due to a chance mutation, that has no effect on enzyme function and is not deleterious, could be passed on to all of the OXA-51-like enzymes that have since evolved from this ancestral sequence. This would result in a

large percentage of OXA-51-like enzymes differing from the consensus sequence at that particular amino-acid position without this position playing any role in the activity of the enzyme. It is possible that variation at amino-acid positions at which a high percentage of OXA-51-like enzymes differ from the consensus sequence, such as at positions 36 and 107, is due to such stochastic effects.

Despite the limitations of the structural models, variations at five amino-acid positions were identified that may have an effect on the activity of the OXA-51-like enzymes. These five amino-acid positions are all found within or very near to the active site cleft of the enzyme. Firstly, amino-acid variation is seen at positions 129 and 130. Analysis of the crystal structure of OXA-40 suggested that the binding of carbapenems was assisted through van der Waals interactions between the methyl group of the carbapenem and the non-polar valine residue at position 130. Additionally it has been suggested that the presence of the non-polar side chain of the valine residue at position 130, which is found in many class D β-lactamases, may be a contributory factor to the broad substrate specificity of these enzymes (Santillana *et al.*, 2007). In the OXA-51-like structural models the residues that take up the synonymous position to valine-130 are at position 129. The variations seen at position 129 within the OXA-51-like enzymes are between a valine, leucine or isoleucine residue. These three residues all have non-polar side chains, and so their substitutions are unlikely to have a role in changing the chemistry of the enzyme-substrate interaction. However, the side chains of the three residues do have different sizes and conformations which may play a role in altering substrate specificity or affinity through changing the shape and size of the end of the cleft.

The effect of changes at position 130 in the OXA-51-like enzymes is less clear. In the OXA-40 structure, there is a proline residue at the synonymous 131 position. The enzyme OXA-66 also has a proline at this position, but some of the other OXA-66-like enzymes have substituted it for a leucine, serine or glutamine residue. Due to the relative inflexibility of proline residues, it is likely that the loss of a proline at this position will allow greater movement of the surrounding residues. This includes the important residue at position 129 discussed above. As the properties of the residues that replace the proline are varied, it is likely that it is the loss of the proline residue to increase branch flexibility, rather than the gaining of a particular property from another residue, that is the reason for the changes.

The third amino-acid position within the active site where variation is seen is position 167. The most common variation at this site is between a leucine and a valine. In the OXA-40 model, there are interactions in the active site whereby the active site serine at position 81 (synonymous with serine 80 in the OXA-51-like enzymes) forms a weak hydrogen bond with a lysine residue at position 84 (position 83 in OXA-51-like enzymes), which itself forms a hydrogen bond with the side chain of a tryptophan residue at position 167 (position 166 in OXA-51-like enzymes) (Santillana *et al.*, 2007). As the chemical properties of leucine and valine are not dissimilar, variation between these two residues in the OXA-51-like enzymes at position 167 is likely to have an effect on the activity of the enzyme through changing the shape of the active site cleft, rather than through biochemical means. It may also have effects such as disrupting the hydrogen bond between the lysine 83 and tryptophan 166 residues. In one enzyme, currently unnamed with the accession

number EU255296, the leucine 167 is replaced with a glutamine residue. The substitution of a hydrophobic amino-acid for a polar one within the active site is likely to have a significant effect on the substrate profile and activity of the enzyme.

The final two of the five amino-acid positions that were identified as potentially affecting the active site of the enzyme are position 168 and 225. The effects of the variations at these positions are difficult to tell from the structural models as there is little actual structural variation evident. Changes at position 168 are between valine, alanine and methionine, which are all hydrophobic (though alanine only slightly), and as such variation between them is unlikely to result in any major changes in the chemical interactions surrounding them. However, they are adjacent to the active site residue 167, and it may be that changes at position 168 alter the conformations that the residue at position 167 is able to adopt. At position 225 residues vary between asparagine and aspartate. In the OXA-40 model shown in figure 37, the loop connecting the β4 and β5 strands is closer to the active site cleft than in the other OXA-type enzymes that have had their structure solved, and it is thought that the stability of the β4-β5 loop is contributed to by a hydrophobic core formed by valine residues at positions 78, 225 and 229, and a tryptophan at 231 (positions 77, 224, 228 and 230 in the OXA-51-like enzymes) (Santillana *et al*., 2007). In the OXA-51-like enzymes, at the end of the β4 strand a much bulkier tryptophan at position 222 replaces the methionine at the synonymous position in OXA-40, and next to this in the β4-β5 loop a much bulkier aspartate residue at position 223 replaces the glycine found in OXA-40 (figure 38). The large differences in the size and conformation of the residues at the end of the β4 strand and the start of the β4-β5 loop between OXA-

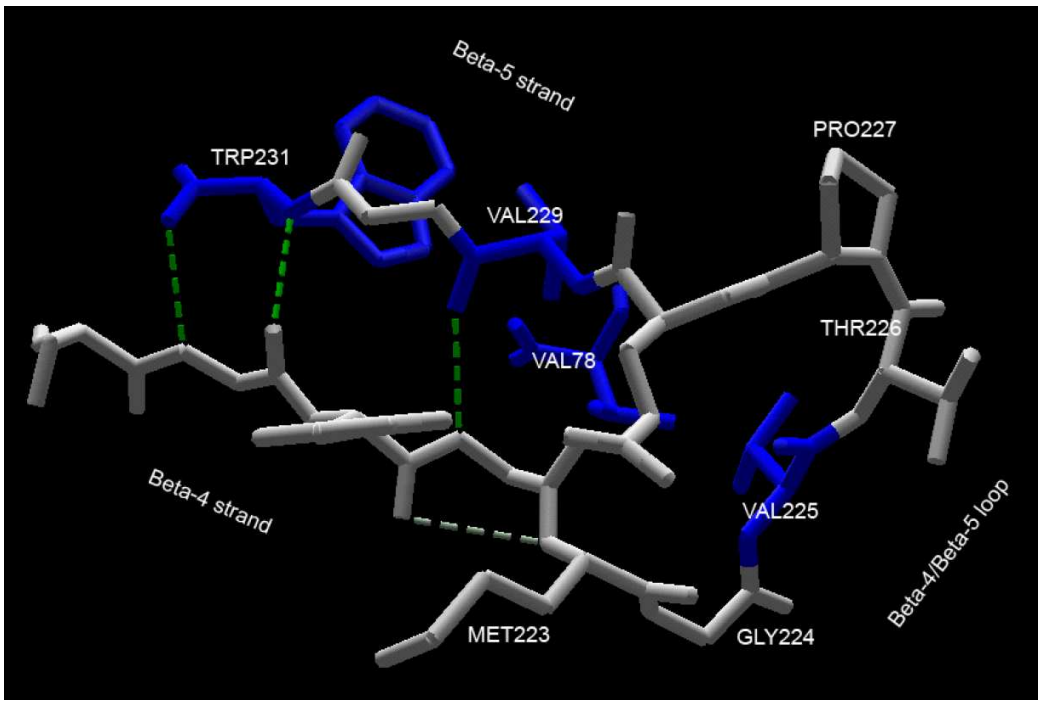

Figure 37: OXA-40 structure showing the β4 and β5 strands and their connecting loop. Residues forming hydrophobic core are blue. Dashed lines are hydrogen bonds. Structure was visualised in DeepView.

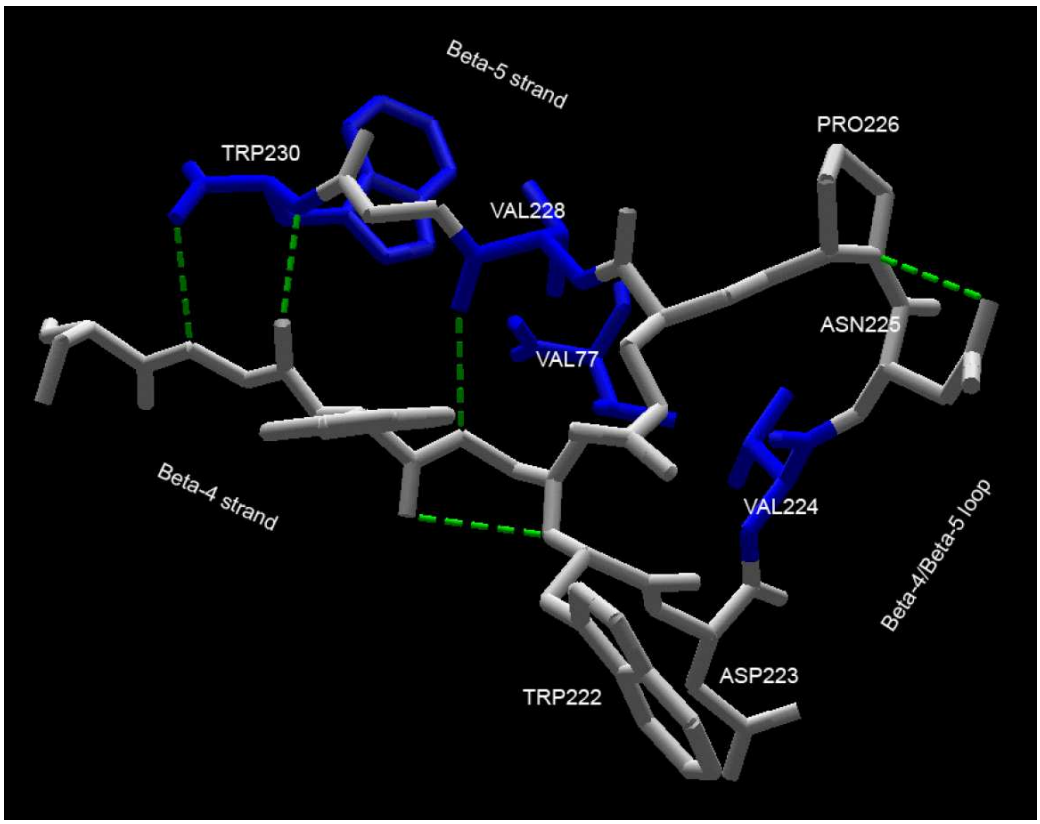

Figure 38: OXA-66 structure showing the β4 and β5 strands and their connecting loop. Residues forming hydrophobic core are blue. Dashed lines are hydrogen bonds. Structure was visualised in DeepView.

40 and the OXA-51-like enzymes is likely to result in differences in the activities of the enzymes. Interestingly it should be noted that in OXA-79, the tryptophan at position 222 has been substituted for a glycine, which is likely to have an effect on the function of the active site. In some of the OXA-51-like enzymes, an asparagine residue is found at position 225, and this may form a hydrogen bond with the adjacent proline 226 that may assist in stabilising the loop. Other OXA-51-like enzymes have an aspartate residue at position 225, which is not shown in the model to form any hydrogen bonds which may make the loop more flexible. The limitations of the models are such that no major structural changes are seen due to differences at position 225. However, it has been suggested that changes in the shape and orientation of the β4-β5 loop may contribute to determining the specificity of the OXA-type β-lactamases for various different β-lactam antibiotics. It is likely that the differences between OXA-40 and the OXA-51-like enzymes in this region of the structure will explain differences in the substrate profiles of the two enzyme groups. Differences at position 225 within the OXA-51-like enzymes may result in subtle differences in the enzyme structure but an accurate crystal structure for this enzyme family is required to examine more closely the effects of this variation.

It is interesting to note that of the five amino-acid positions examined in this study at which variation is thought may have an effect on the activity of the OXA-51-like enzymes, all the variation except that at position 225 is found to occur between enzymes whose genes are at the very tip of their respective branches in the *bla*$_{\text{OXA-51-like}}$ gene tree. For example, variation at position 129 is seen in OXA-83 and OXA-131, branch-tip enzymes in the *bla*$_{\text{OXA-66}}$ clade, and OXA-110, at the tip of the

bla$_{\text{OXA-69}}$ clade (figure 39). This suggests that much of this variation has arisen recently and that this may have been in response to antibiotic pressure. Therefore it is possible that these more recently evolved enzymes represent either altered or extended-spectrum variants of the OXA-51-like enzymes. An analogous situation appears to be occurring with the chromosomal class C AmpC enzymes in *Escherichia coli* and *Pseudomonas aeruginosa*. Chromosomal AmpC enzymes from isolates that predate the antibiotic era are able to hydrolyse the early-generation cephalosporins and appear to have evolved in response to the naturally-occurring cephalosporins produced by environmental organisms (Barlow and Hall, 2002). However, recently variants of the AmpC enzymes in both *E. coli* and *P. aeruginosa* have been detected that appear to have broadened their spectrum of activity to include the later-generation cephalosporins and in some cases the carbapenems (Mammeri *et al*., 2008, Mammeri *et al*., 2006, Rodriguez-Martinez *et al*., 2009). These enzymes have been named extended-spectrum AmpCs (ESACs). It is tempting to speculate that similar antibiotic pressures have resulted in evolution in *A. baumannii* to form extended-spectrum OXA-51-like enzymes. Continued work to analyse the different kinetic properties of these enzymes is required to determine if this is the case.

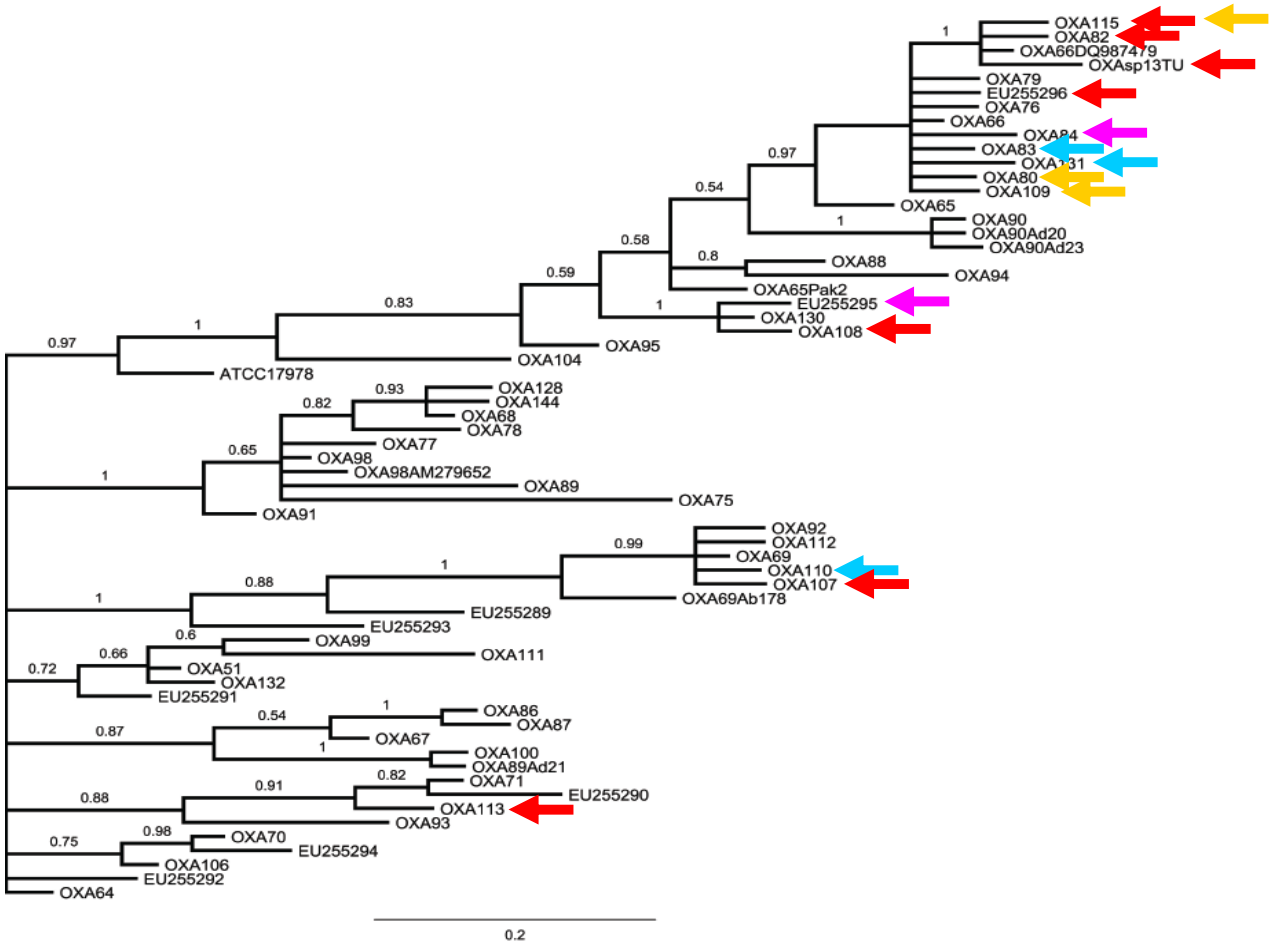

Figure 39: *bla*$_{\text{OXA-51-like}}$ genes encoding enzymes with amino-acid substitutions likely to effect enzyme activity. Bayesian *bla*$_{\text{OXA-51-like}}$ phylogeny is as described in figure 25. Blue arrows, enzymes varying at position 129; orange arrows, enzymes varying at position 130; red arrows, enzymes varying at position 167; pink arrows, enzymes varying at position 168.

In contrast, the origin of variation at position 225 appears to be found much further towards the root of the *bla*$_{\text{OXA-51-like}}$ gene tree, with certain clades containing genes for enzymes with an asparagine, while others have an aspartate. If there is an effect on enzyme function due to variation at this position it is likely to be one with a broader scope than one such as increased carbapenem hydrolysis.

4.8 Summary.

This study aimed to investigate in detail the OXA-51-like enzymes of *A. baumannii*: how they are related to carbapenem resistance within isolates, how isolate epidemiology corresponds with *bla*$_{\text{OXA-51-like}}$ gene content, and how the OXA-51-like enzymes have evolved and what the possible consequences of the variation within their sequences may be. During the course of the study several interesting and important results have been found. Firstly, isolates belonging to SG1 and SG2 appear to be the most successful, certainly across Europe, and within these isolates it is the *bla*$_{\text{OXA-66}}$ and *bla*$_{\text{OXA-69}}$ genes that are most regularly identified. Most of these isolates possess an IS*Aba1* element, which lends weight to the suggestion that the presence of IS*Aba1* within an isolate is a large contributing factor to increased success. For the main sequence groups, SG1, SG2 and SG3, the *bla*$_{\text{OXA-51-like}}$ gene that an isolate carries correlates with the sequence group they belong to. This means that for these sequence groups, identification of the *bla*$_{\text{OXA-51-like}}$ gene an isolate carries is sufficient to determine which lineage the isolate belong to. There is also evidence that there may be further large sequence groups of isolates. Further development of the sequence grouping scheme that confirmed this would mean that many if not all *A. baumannii* could be assigned to an epidemic lineage based upon the *bla*$_{\text{OXA-51-like}}$

gene they contain. While PFGE has been shown to be inappropriate for use with *A. baumannii* isolates collected over more than a short period of time, MLST has been shown to correlate both with the sequence grouping results and with $bla_{\text{OXA-51-like}}$ gene content, assuming appropriate 'housekeeping' alleles are utilised. This further strengthens the argument that the determination of the $bla_{\text{OXA-51-like}}$ gene of an isolate could serve as a useful epidemiological tool.

It had been argued that the $bla_{\text{OXA-51-like}}$ genes of *A. baumannii* are 'housekeeping' genes (Whitman *et al*., 2008). This has been shown to be incorrect, as the variation seen within the genes and their evolution is akin to other well-known β-lactamases such as the TEM, SHV and CTX-M families than to 'housekeeping' genes. It has been shown that isolates in this study that possess an IS*Aba1* element upstream of their $bla_{\text{OXA-51-like}}$ gene have carbapenem MICs that are not statistically significantly different to those of isolates that possess an acquired OXA-type β-lactamase of the OXA-23, OXA-40 or OXA-58 families. Additionally, the presence of IS*Aba1* upstream of a $bla_{\text{OXA-51-like}}$ gene is correlated with a greatly increased ability of the isolate to survive carbapenem challenge, even at concentrations far above the carbapenem MIC for the isolate. These findings indicate that in the isolates in this study the expression of certain OXA-51-like enzymes is as important a threat to carbapenem use as the acquired OXA enzymes. This is of great concern, as it presents the scenario that all *A. baumannii* have the potential to become resistant to the carbapenems without needing to acquire additional β-lactamases.

Analysis of the sequences of 'housekeeping' genes and *bla*$_{\text{OXA-51-like}}$ genes shows that the species *A. baumannii* consists of several phylogenetically distinct lineages that appear to be evolving at different rates. Additionally there is evidence that the species has undergone successive selective sweeps during the past. Within the major enzyme groups, the enzymes that are closely related to OXA-66, OXA-69 and OXA-98 seem to have evolved most recently, and it is within such enzymes that amino-acid variation at positions 129, 130 and 167 occur, which may have an affect on the activity of the enzyme, with the possibility that the variation has arisen in response to antibiotic use.

In order to test the hypothesis posed for this project, a series of five questions were asked. The first question asked:

- How prevalent are the *bla*$_{\text{OXA-51-like}}$ genes, and are all of the various alleles distributed equally?

This has been answered by showing that the *bla*$_{\text{OXA-51-like}}$ genes appear to be universal within *A. baumannii*, but that there are distinct biases in the distribution of the alleles. The reason for this answers the second question, which asked:

- Are particular *bla*$_{\text{OXA-51-like}}$ alleles associated with certain strains, and are certain strains more resistant to the carbapenems than others?

It has been demonstrated that certain successful lineages of *A. baumannii* are associated with particular groups of closely related *bla*$_{\text{OXA-51-like}}$ genes. This study did not find that particular lineages of *A. baumannii* were likely to be significantly more resistant to the carbapenems than others. However, the third question asked:

- Can insertion sequences be found in association with all *bla*$_{\text{OXA-51-like}}$ genes?

The data show that it is isolates that carry a *bla*$_{\text{OXA-66-like}}$, *bla*$_{\text{OXA-69-like}}$, or *bla*$_{\text{OXA-51}}$ gene that are associated with an IS*Aba1* element, and the IS*Aba1* element could not be induced to insert upstream of the *bla*$_{\text{OXA-51-like}}$ genes, suggesting that under the conditions tested, not all *A. baumannii* are conducive to IS*Aba1* insertion. This also partially relates to question four, which asked:

- Can susceptible *A. baumannii* acquire carbapenem resistance conferred by the *bla*$_{\text{OXA-51-like}}$ alleles under selective pressure?

For the isolates studied here this was shown not to be the case, either by IS*Aba1*-conferred increased expression or changes in the *bla*$_{\text{OXA-51-like}}$ genes themselves. The final question asked:

- Do all of the OXA-51-like enzymes show structural similarities indicating a shared ability to hydrolyse similar substrates?

The answer to this is that while many of the OXA-51-like enzymes appear to have similar structures, the recently evolved enzymes have amino-acid changes that may affect the hydrolytic properties of the enzymes, altering their substrate profiles.

Together the results of this study demonstrate that the OXA-51-like enzymes are an important feature to consider when studying *A. baumannii*. The ability to use their identification as a proxy for more expensive or time-consuming typing techniques shows great potential for their utilisation in epidemiological studies. Additionally, the presence of some of the enzymes is correlated with clinically significant levels of resistance to the carbapenems, and association with epidemic lineages. Therefore the identification of the *bla*$_{\text{OXA-51-like}}$ gene that an isolate carries provides important information indicating the likelihood of whether treatment with a carbapenem will be

successful. Finally, the *bla*$_{\text{OXA-51-like}}$ genes appear to be continually evolving, and the monitoring of this process to identify enzyme variants with increased substrate spectrums or higher levels of activity is crucial if we are to preserve and use effectively the few remaining antibiotic treatment options that are available for this multiresistant pathogen.

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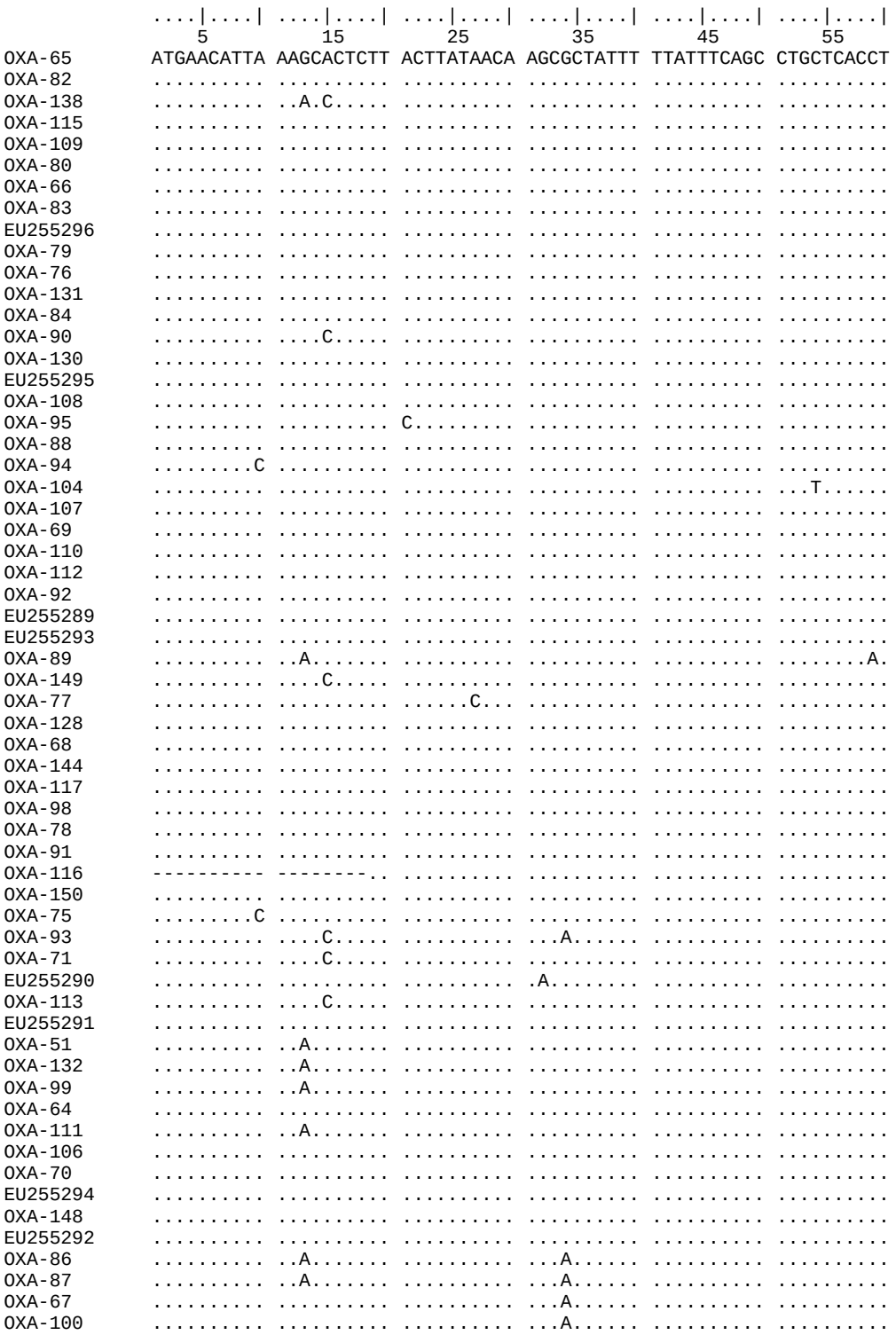
Appendix A.

Nucleotide and Amino-acid sequence alignments.

1. Nucleotide alignment for all 60 *bla*$_{\text{OXA-51-like}}$ genes. Substitutions are labelled with respect to *bla*$_{\text{OXA-65}}$.



```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    65         75         85         95        105        115
OXA-65          TATATAGTGA CTGCTAATCC AAATCACAGT GCTTCAAAAT CTGATGAAAA AGCAGAGAAA
OXA-82          .......... .......... .........C .......... ......T... ..........
OXA-138         .......... .......... .........C .......... ......T... ..........
OXA-115         .......... .......... .........C .......... ......T... ..........
OXA-109         .......... .......... .........C .......... ......T... ..........
OXA-80          .......... .......... .........C .......... ......T... ..........
OXA-66          .......... .......... .........C .......... ......T... ..........
OXA-83          .......... .......... .........C .......... ......T... ..........
EU255296        .......... .......... .........C .......... ......T... ..........
OXA-79          .......... .......... .........C .......... ......T... ..........
OXA-76          .......... .......... .........C .......... ......T... ..........
OXA-131         .......... .......... .........C .......... ......T... ..........
OXA-84          .......... .......... .........C .......... ......T... ..........
OXA-90          .......... .......... .........C .......... .......... ..........
OXA-130         .......... ..A....... .........C .......... .......... ..........
EU255295        .......... ..A....... .........C .......... .......... ..........
OXA-108         .......... ..A....... .........C .......... .......... ..........
OXA-95          .......... .......... .........C .......... .......... ..........
OXA-88          .......... .......... .........C .......... .....A.... ..........
OXA-94          .........T .......... .......... .......... .......... ..........
OXA-104         .......... .......... .........C .......... .......... ..........
OXA-107         .......... .......... .......... .......... .......C.. ..........
OXA-69          .......... .......... .......... .......... .......C.. ..........
OXA-110         .......... .......... .......... .......... .......C.. ..........
OXA-112         .......... .......... .......... .......... .......C.. ..........
OXA-92          .......... .......... .......... .......... .......C.. ..........
EU255289        .......... .......... .......... .......... .......C.. ..........
EU255293        .......... .......... .......... .......... .......C.. ..........
OXA-89          .........T .......... .......... .......... .......... ..........
OXA-149         .........T .......... .......... .......... .......... ..........
OXA-77          .........T .......... .......... .......... .......... ..........
OXA-128         .........T .......... .......... .......... .......... ..........
OXA-68          .........T .......... .......... .......... .......... ..........
OXA-144         .........T .......... .......... .......... .......... ..........
OXA-117         .........T .......... .......... .......... .......... ..........
OXA-98          .........T .......... .......... .......... .......... ..........
OXA-78          .........T .......... .......... .......... .......... ..........
OXA-91          .........T .......... .......... .......... .......... ..........
OXA-116         .........T .......... .......... .......... .......C.. ..........
OXA-150         .........T .......... .......... .......... .......... ..........
OXA-75          .......... .......... ....T..... .......... .......... ..........
OXA-93          .......... .......... .........C .......... .......... ..........
OXA-71          .......... .......... .........C .......... .......... ..........
EU255290        .......... .......... .........C .......... .......... ..........
OXA-113         .......... .......... .........C .......... .......... ..........
EU255291        .......... .......... .........C .......... .......... ..........
OXA-51          .......... .......... .........C .......... .......... ..........
OXA-132         .......... .......... .........C .......... .......... ..........
OXA-99          .......... .......... .........C .......... .......... ..........
OXA-64          .......... .......... .........C .......... .......... ..G.......
OXA-111         .........T .......... .......... .......... .......... ..........
OXA-106         .......... .......... .........C .......... .....A.... ..........
OXA-70          .......... .......... .........C .......... .....A.... ..........
EU255294        .......... .......... .........C .......... .....A.... ..........
OXA-148         .......... .......... .........C .......... .....A.... ..........
EU255292        .......... .......... .........C .......... .....A.... ..........
OXA-86          .......... .......... .........C A........ .......... ..........
OXA-87          .......... .......... .........C A........ .......... ..........
OXA-67          .......... .......... .........C A........ .......... ..........
OXA-100         .......... .......... .........C A........ .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   125        135        145        155        165        175
OXA-65          ATTAAAAATT TATTTAACGA AGCACACACT ACGGGTGTTT TAGTTATCCA ACAAGGCCAA
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-66          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-90          .......... .......... .......... .......... .......... ..........
OXA-130         .......... .......... .......... .......... .......... ..........
EU255295        .......... .......... .......... .......... .......... ..........
OXA-108         .......... .......... .......... .......... .......... ..........
OXA-95          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-94          .......... .......... .......... .......... .......... ..........
OXA-104         .......... .......... .......... .......... .......... ..........
OXA-107         .......... .......... .......... .......... .......... T.....T...
OXA-69          .......... .......... .......... .......... .......... T.....T...
OXA-110         .......... .......... .......... .......... .......... T.....T...
OXA-112         .......... .......... .......... .......... .......... T.....T...
OXA-92          .......... .......... .......... .......... .......... T.....T...
EU255289        .......... .......... .......... .......... .......... T.....T...
EU255293        .......... .......... .......... .......... .......... T.....T...
OXA-89          .......... .......... .......... .......... .......... ..........
OXA-149         .......... .......... .......... .......... .......T.. ..........
OXA-77          .......... .......... .......... .......... .......... ..........
OXA-128         .......... .......... .......... .......... .......... ..........
OXA-68          .......... .......... .......... .......... .......... ..........
OXA-144         .......... .......... .......... .......... .......... ..........
OXA-117         .......... .......... .......... .......... .......... ..........
OXA-98          .......... .......... .......... .......... .......... ..........
OXA-78          .......... .......... .......... .......... .......... ..........
OXA-91          .......... .......... .......... .......... .......... ..........
OXA-116         .......... .......... .......... .......... .......... ..........
OXA-150         .......... .......... .......... .......... .......T.. ..........
OXA-75          .......... .......... .......... .......... .......... ..........
OXA-93          .......... .......... .......... ........C. .......... ..........
OXA-71          .......... .......... .......... ........C. .......... ..........
EU255290        .......... .......... .......... ........C. .......... ..........
OXA-113         .......... .......... .......... ........C. .......... ..........
EU255291        .......... .......... ..T....... .......... .......... ..........
OXA-51          .......... .......... ..T....... .......... .......... ..........
OXA-132         .......... .......... ..T....... .......... .......... ..........
OXA-99          .......... .......... ..T....... .......... .........G ..........
OXA-64          .......... .......... .......... .......... .......... ..........
OXA-111         .......... .......... .......... .......... .......... ..........
OXA-106         .......... .......... .......... .......... .......... ..........
OXA-70          .......... .......... .......... .......... .......... ..........
EU255294        .......... .......... .......... .......... .......... ..........
OXA-148         .......... .......... .......... .......... .......... ..........
EU255292        .......... .......... .......... .......... .......... ..........
OXA-86          .......... .......... .......... .......... .......... ..........
OXA-87          .......... .......... .......... .......... .......... ..........
OXA-67          .......... .......... .......... .......... .......... ..........
OXA-100         .......... .......... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   185        195        205        215        225        235
OXA-65          ACTCAACAAA GCTATGGTAA TGATCTTGCT CGTGCTTCGA CCGAGTATGT ACCTGCTTCG
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-66          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-90          .......... .......... .......... .......... .......... ..........
OXA-130         .......... .......... .......... .......... .......... ..........
EU255295        .......... .......... .......... .......... .......... ..........
OXA-108         .......... .......... .......... .......... .......... ..........
OXA-95          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-94          .......... .......... .......... .......... .......... ..........
OXA-104         .......... .......... .......... .......... .......... ..........
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-69          .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........
OXA-92          .......... .......... .......... .......... .......... ..........
EU255289        .......... .......... .......... .......... .......... ..........
EU255293        .......... .......... .......... .......... .......... ..........
OXA-89          .......... .......... .......... .....A.... .......... ..........
OXA-149         .......... .......... .......... .......... .......... ..........
OXA-77          .......... .......... .......... .......... .......... ..........
OXA-128         .......... .......... ..T....... .......... .......... ..........
OXA-68          .......... .......... .......... .......... .......... ..........
OXA-144         .......... .......... .......... .......... .......... ..........
OXA-117         .......... .......... .......... .......... .......... ..........
OXA-98          .......... .......... .......... .......... .......... ..........
OXA-78          .......... .......... .......... .......... .......... ..........
OXA-91          .......... .......... .......... .......... .......... ..........
OXA-116         .......... .......... .......... .......... .......... ..........
OXA-150         .......... .......... .......... .......... .......... ..........
OXA-75          .......... .......... .......... .......... .......... ..........
OXA-93          .......... .......... .......... .......... .......... ..........
OXA-71          .......... .......... .......... .......... .......... ..........
EU255290        .......... .......... .......... .......... .......... ..........
OXA-113         .......... .......... .......... .......... .......... ..........
EU255291        .......... .......... .......... .......... .......... ..........
OXA-51          .......... .......... .......... .......... .......... ..........
OXA-132         .......... .......... .......... .......... .......... ..........
OXA-99          .......... .......... .......... .......... .......... ..........
OXA-64          .......... .......... .......... .......... .......... ..........
OXA-111         .......... .......... .......... .....A.... .......... ..........
OXA-106         .......... .......... .......... .......... .......... ..........
OXA-70          .......... .......... .......... .......... .......... ..........
EU255294        .......... .......... .......... .......... .......... ..........
OXA-148         .......... .......... .......... .......... .......... ..........
EU255292        .......... .......... .......... .......... .......... ..........
OXA-86          .......... .......... .......... .......... T......... ..........
OXA-87          .......... .......... .......... .......... T......... ..........
OXA-67          .......... .......... .......... .......... .......... ..........
OXA-100         .......... .......... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   245        255        265        275        285        295
OXA-65          ACCTTCAAAA TGCTTAATGC TTTGATCGGC CTTGAGCACC ATAAGGCAAC CACCACAGAA
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-66          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-90          .......... .......... .......... .......... .......... ..........
OXA-130         .......... .......... .......... .......... .......... ..........
EU255295        .......... .......... .......... .......... .......... ..........
OXA-108         .......... .......... .......... .......... .......... ..........
OXA-95          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-94          .......... .......... .......... .......... .......... ..........
OXA-104         .......... .......... .......... .......... .......... ..........
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-69          .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........
OXA-92          .......... .......... .......... .......... .......... ..........
EU255289        .......... .......... .......... .......... .......... ..........
EU255293        .......... .......... .......... .......... .......... ..........
OXA-89          .......... .......... .......... .......... .......... ...T......
OXA-149         .......... .......... .......... .......... .......... ...T......
OXA-77          .......... .......... .......... .......... .......... ...T......
OXA-128         .......... .......... .......... .......... .......... ...T......
OXA-68          .......... .......... .......... .......... .......... ...T......
OXA-144         .......... .......... .......... .......... .......... ...T......
OXA-117         .......... .......... .......... .......... .......... ...T......
OXA-98          .......... .......... .......... .......... .......... ...T......
OXA-78          .......... .......... .......... .......... .......... ...T....C.
OXA-91          .......... .......... .......... .......... .......... ...T......
OXA-116         .......... .......... .......... .......... .......... ...T......
OXA-150         .......... .......... .......... .......... .......... ...T......
OXA-75          .......... .......... ......A... .......... .......... ...T......
OXA-93          .......... .......... .......A.. .......... .......... ..........
OXA-71          .......... .......... .......... .......... .....A.... ..........
EU255290        .......... .......... .......... .......... .....A.... ..........
OXA-113         .......... .......... .......... .......... .....A.... ..........
EU255291        .......... .......... .......... .......... .......... ..........
OXA-51          .......... .......... .......... .......... .......... ..........
OXA-132         .......... .......... .......... .......... .......... ..........
OXA-99          .......... .......... .......... .......... .......... ..........
OXA-64          .......... .......... .......... .......... .......... ..........
OXA-111         .......... .......... .......... .......... .......... ..........
OXA-106         .......... .......... .......... .......... .......... ..........
OXA-70          .......... .......... .......... .......... .......... ..........
EU255294        .......... .......... .......... .......... .......... ..........
OXA-148         .......... .......... .......... .......... .........G ..........
EU255292        .......... .......... .......... .......... .......... ..........
OXA-86          .......... .......... .......... .......... .......... ..........
OXA-87          .......... .......... .......... .......... .......... ..........
OXA-67          .......... .......... .......... .......... .......... ..........
OXA-100         .......... .......... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   305        315        325        335        345        355
OXA-65          GTATTTAAGT GGGATGGTAA AAAAAGGTTA TTCCCAGAAT GGGAAAAGGA CATGACCCTA
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-66          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-90          .......... .......... .......... .......... .......... ..........
OXA-130         .......... .......... .......... .......... .......... ..........
EU255295        .......... .......... .......... .......... .......... ..........
OXA-108         .......... .......... .......... .......... .......... ..........
OXA-95          .......... .......... .......C.. .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-94          .......... .......... .......... .......... .......... ..........
OXA-104         .......... .......... .......C.. .......... ........A. ..........
OXA-107         ........A. .......GG. .......C.. .......... ........A. ..........
OXA-69          ........A. .......GG. .......C.. .......... ........A. ..........
OXA-110         ........A. .......GG. .......C.. .......... ........A. ..........
OXA-112         ........A. .......GG. .......C.. .......... ........A. ..........
OXA-92          ........A. .......GG. .......C.. .......... ........A. ..........
EU255289        A......... ....C..GC. .......C.G .......... .......... ..........
EU255293        .......... ..A.C..GC. .......C.G .......... .......... ..........
OXA-89          .......... ....C..GC. .......C.. .......... ........A. ..........
OXA-149         .......... ....C..GC. .......C.. .......... ........A. ..........
OXA-77          .......... ....C..GC. .......C.. .......... ........A. ..........
OXA-128         .......... ....C..GC. .......C.. .......... ........A. ..........
OXA-68          .......... ....C..GC. .......C.. .......... ........A. ..........
OXA-144         .......... ....C..GC. .......C.. .......... ........A. ..........
OXA-117         .......... ....C..GC. .......C.. .......... ........A. ..........
OXA-98          .......... ....C..GC. .......C.. .......... ........A. ..........
OXA-78          .......... ....C..GC. .......C.. .......... ........A. ..........
OXA-91          .......... ....C..GC. .......C.. .......... ........A. ..........
OXA-116         .......... ....C..GC. .......C.. .......... ........A. ..........
OXA-150         .......... ....C..GC. .......C.. .......... ........A. ..........
OXA-75          .......... ....C..GC. .......C.. .......... ........A. ..........
OXA-93          .......... ....C..GC. .......C.. .......... .......... ..........
OXA-71          .......... ....C..GC. .......C.. .......... .......... ..........
EU255290        .......... ....C..GC. .......C.. .G........ .......... ..........
OXA-113         .......... ....C..GC. .......C.. .......... .......... ..........
EU255291        .......... ....C..GC. .......C.. .......... .......... ..........
OXA-51          .......... ....C..GC. .......C.. .......... .......... ..........
OXA-132         .......... ....C..GC. .......C.. .......... .......... ..........
OXA-99          .......... ....C..GC. .......C.. .......... .......... ..........
OXA-64          .......... ....C..GC. .......C.. .......... .......... ..........
OXA-111         .......... ....C..GC. .......C.. .......... .......... ..........
OXA-106         .......... ..A.C..GC. .......C.G .......... .......... ..........
OXA-70          .......... ..A.C..GC. .......C.G .......... .......... ..........
EU255294        .......... ..A.C..GC. .......C.G .......... .......... ..........
OXA-148         .......... ..A.C..GC. .......C.G .......... .......... ..........
EU255292        .......... ..A.C..GC. .......C.G .......... .......... ..........
OXA-86          A......... ....C..GC. .......C.G .......... .......... ..........
OXA-87          A......... ....C..GC. .......C.G .......... .......... ..........
OXA-67          A......... ....C..GC. .......C.G .......... .......... ..........
OXA-100         A......... ....C..GC. .......C.G .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   365        375        385        395        405        415
OXA-65          GGCGATGCCA TGAAAGCTTC CGCTATTCCA GTTTATCAAG ATTTAGCTCG TCGTATTGGA
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......T.. .......... .......... ..........
OXA-109         .......... .......... .......A.. .......... .......... ..........
OXA-80          .......... .......... .......T.. .......... .......... ..........
OXA-66          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... ....C..... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... ....G..... .......... .......... ..........
OXA-84          .......... .......... ......T... .......... .......... ..........
OXA-90          .......... .......... .......... .......... .......... ..........
OXA-130         .......... .......... .......... .......... .......... ..........
EU255295        .......... .......... .......... .......... .......... ..........
OXA-108         .......... .......... .......... .......... .......... ..........
OXA-95          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .........G .......... .......... ..........
OXA-94          .......... .......... .........G .......... .......... ..........
OXA-104         ........T. .......... .........G .......... .......... ..........
OXA-107         ........T. .......... .........G .......... .......... ..........
OXA-69          ........T. .......... .........G .......... .......... ..........
OXA-110         ........T. .......... ....C....G .......... .......... ..........
OXA-112         ........T. .......... .........G .......... .......... ..........
OXA-92          ........T. .......... .........G .......... .......... ..........
EU255289        ..T.....T. .......... .........G .......... .......... ..........
EU255293        ........T. .......... .........G .......... .......... ..........
OXA-89          ........T. .......... .........G .......... .......... ..........
OXA-149         ........T. .......... .........G .......... .......... ..........
OXA-77          ........T. .......... .........G .......... .....C.... ..........
OXA-128         ........T. .......... .........G .......... .......... ..........
OXA-68          ........T. .......... .........G .......... .......... ..........
OXA-144         ........T. .......... .........G .......... .......... ..........
OXA-117         ........T. .......... .........G .......... .......... ..........
OXA-98          ........T. .......... .........G .......... .......... ..........
OXA-78          ........T. .......... .........G .......... .......... ..........
OXA-91          ........T. .......... .........G .......... .......... ..........
OXA-116         ........T. .......... .........G .......... .......... ..........
OXA-150         ........T. .......... .........G .......... .......... ..........
OXA-75          ........T. .......... .........G .......... .......... ..........
OXA-93          .....C..T. .......... .........G .......... .......... ..........
OXA-71          .....C..T. .......... .........G .......... .......... ..........
EU255290        .....C..T. .......... .........G .......... .......... ..........
OXA-113         .....C..T. .......... .........G .......... .......... ..........
EU255291        ........T. .......... .........G .......... .......... ..........
OXA-51          ........T. .......... .........G .......... .......... ..........
OXA-132         ........T. .......... .........G .......... .......... ..........
OXA-99          ........T. .......... .........G .......... .......... ..........
OXA-64          ........T. .......... .........G .......... .......... ..........
OXA-111         ........T. .......... .........G .......... .......... ..........
OXA-106         ........T. .......... .........G .......... .......... ..........
OXA-70          ........T. .......... .........G .......... .......... ..........
EU255294        ........T. .......... .........G .......... .......... ..........
OXA-148         ........T. .......... .........G .......... .......... ..........
EU255292        ........T. .......... .........G .......... .......... ..........
OXA-86          ..T.....T. .......... .........G .......... .......... ..........
OXA-87          ..T.....T. .......... .........G .......... .......... ..........
OXA-67          ..T.....T. .......... .........G .......... .......... ..........
OXA-100         ..T.....T. .......... .........G .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   425        435        445        455        465        475
OXA-65          CTTGAGCTCA TGTCTAAGGA AGTGAAGCGT GTTGGTTATG GCAATGCAGA TATCGGTACC
OXA-82          .......... ....A..... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-66          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-90          .......... .......... .......... .......... .......... ..........
OXA-130         .......... .......... .......... .......... .....A.... ..........
EU255295        .......... .......... .......... .......... .....A.... ..........
OXA-108         .......... .......... .......... .......... .....A.... ..........
OXA-95          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-94          .......... .......... .......... .......... .......... ..........
OXA-104         .....A.... .......T.. .......... .......... .......... ..........
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-69          .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........
OXA-92          .......... .......... .......... .......... .......... ..........
EU255289        .....A.... .......... .......... .......... .......... ..........
EU255293        .....A.... .......T.. .......... .......... ........C. ..........
OXA-89          .....A.... .......T.. .......... .......... .......... ..........
OXA-149         .....A.... .......T.. ........A. .......... .......... ..........
OXA-77          .....A.... .......T.. .......... .......... .......... ..........
OXA-128         .....A.... .......T.. .......... .......... .......... ..........
OXA-68          .....A.... .......T.. .......... .......... .......... ..........
OXA-144         .....A.... .......T.. .......... .......... .......... ..........
OXA-117         .....A.... .......T.. .......... .......... .......... ..........
OXA-98          .....A.... .......T.. .......... .......... .......... ..........
OXA-78          .....A.... .......T.. .......... .......... .......... ..........
OXA-91          .....A.... .......T.. .......... .......... .......... ..........
OXA-116         .....A.... .......T.. .......... .......... .......... ..........
OXA-150         .....A.... .......T.. ........A. .......... .......... ..........
OXA-75          .....A.... .......T.. .......... A......... .......... ..........
OXA-93          .....A.... .......T.. .......... .......... .......... ..........
OXA-71          .....A.... .......... .......... .......... .......... ..........
EU255290        .....A.... .......... .......... .......... .......... ..........
OXA-113         .....A.... .......... .......... .......... .......... ..........
EU255291        .....A.... .......... .......... .......... .......... ..........
OXA-51          .....A.... .......... .......... .......... .......... ..........
OXA-132         .....A.... .......... .......... .......... .......... ..........
OXA-99          .....A.... .......... .......... .......... .......... ..........
OXA-64          .....A.... .......... .......... .......... .......... ..........
OXA-111         .....A.... .......... .......... .......... .......... ..........
OXA-106         .....A.... .......T.. .......... .......... .......... ..........
OXA-70          .....A.... .......T.. .......... .......... .......... ..........
EU255294        .....A.... .......T.. .......... .......... .......... ..........
OXA-148         .....A.... .......T.. .......... .......... .......... ..........
EU255292        .....A.... .......T.. .......... .......... .......... ..........
OXA-86          .....A.... .......... .......... .......... .......... ..........
OXA-87          .....A.... .......... .......... .......... .......... ..........
OXA-67          .....A.... .......... .......... .......... .......... ..........
OXA-100         .....A.... .......... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   485        495        505        515        525        535
OXA-65          CAAGTCGATA ATTTTTGGCT GGTGGGTCCT TTAAAAATTA CTCCTCAGCA AGAGGCACAG
OXA-82          .......... ........G. .......... .......... .......... ..........
OXA-138         .......... ........G. .......... .......... .......... ..........
OXA-115         .......... ........G. .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-66          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
EU255296        .......... .........A .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... ..C....... .......... .......... ..........
OXA-90          .......... .......... A......... .......... .......... ..........
OXA-130         .......... .......... .......... .......... .......... ..........
EU255295        .......... .......... .A........ .......... .......... ..........
OXA-108         .......... ........G. .......... .......... .......... ..........
OXA-95          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-94          .......... .......... .......... .......... .......... ..........
OXA-104         .......... .......... .......... .......... .......... ..........
OXA-107         .......... ........G. .......... C......... .......... ..........
OXA-69          .......... .......... .......... C......... .......... ..........
OXA-110         .......... .......... .......... C......... .......... ..........
OXA-112         .......... .......... .......... C......... .......... ..........
OXA-92          .......... .......... .......... C......... .......... ..........
EU255289        .......... .......... .......... .......... .......... ..........
EU255293        .......... .......... .......... .......... .......... .........A
OXA-89          .......... .......... .......... .......... .......... .........A
OXA-149         .......... .......... .......... .......... .......... .........A
OXA-77          .......... .......... .......... .......... .......... .........A
OXA-128         .......... .......... A......... .......... .......... .........A
OXA-68          .......... .......... A......... .......... .......... .........A
OXA-144         .......... .......... A......... .......... .......... .........A
OXA-117         .......... .......... A......... .......... .......... .........A
OXA-98          .......... .......... .......... .......... .......... .........A
OXA-78          .......... .......... A......... .......... .......... .........A
OXA-91          .......... .......... .......... .......... .......... .........A
OXA-116         .......... .......... .......... .......... .......... ..........
OXA-150         .......... .......... .......... .......... .......... .........A
OXA-75          .......... .......... .......... .......... .......A.. .........A
OXA-93          .......... .......... .......... .......... .......... .........A
OXA-71          .......... .......... .......... .......... .......... .........A
EU255290        .......... .......... .......... .......... .......... .........A
OXA-113         .......... ........G. .......... .......... .......... .........A
EU255291        .......... .......... .......... .......... .......... ..........
OXA-51          .......... .......... .......... .......... .......... ..........
OXA-132         .......... .......... .......... .......... .......... ..........
OXA-99          .......... .......... .......... .......... .......... ..........
OXA-64          .......... .......... .......... .......... .......... ..........
OXA-111         .......... .......... ......C... .......... .......... ..........
OXA-106         .......... .......... .......... .......... .......... .........A
OXA-70          .......... .......... .......... .......... .......... .........A
EU255294        .......... .......... .......... .......... .......... .........A
OXA-148         .......... .......... .......... .......... .......... .........A
EU255292        .......... .......... .......... .......... .......... ..........
OXA-86          .......... .......... .......... .......... .......... ..........
OXA-87          .......... .......... .......... .......... .......... ..........
OXA-67          .......... .......... .......... .......... .......... ..........
OXA-100         .......... .......... .......... .......... .G........ .........A
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   545        555        565        575        585        595
OXA-65          TTTGCTTACA AGCTAGCTAA TAAAACGCTT CCATTTAGCC AAAAAGTCCA AGATGAAGTG
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-66          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-90          .......... .......... .......... .......... .......... ..........
OXA-130         .......... .......... .......... .......... .......... ..........
EU255295        .......... .......... .......... .......... .......... ..........
OXA-108         .......... .......... .......... .......... .......... ..........
OXA-95          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-94          .......... .......... .......... .......... .......... ..........
OXA-104         ........T. .......... .......... .......... .......... ..........
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-69          .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........
OXA-92          .......... .......... .......... .......... .......... ..........
EU255289        .......... .......... .......... .......... .......... ..........
EU255293        .......... .......... .......... .......... .......... ..........
OXA-89          ...A...... .......... .......... .......... .......... ..........
OXA-149         .......... .......... .......... .......... C......... ..........
OXA-77          .......... .......... .......... .......... .......... ..........
OXA-128         .......... .......... .......... .......... ..G....... ..........
OXA-68          .......... .......... .......... .......... ..G....... ..........
OXA-144         .......... .......... .......... .......... ..G....... ..........
OXA-117         .......... .......... .......... .......... .......... ..........
OXA-98          .......... .......... .......... .......... .......... ..........
OXA-78          .......... .......... .......... .......... .......... ..........
OXA-91          .......... .......... .......... .......... .......... ..........
OXA-116         .......... .......... .......... .......... .......... ..........
OXA-150         .......... .......... .......... .......... .......... ..........
OXA-75          .......... .......... .......... .......... .......... ..........
OXA-93          .......... .......... .......... .......... .......... ..........
OXA-71          .......... .......... .......... .......... C......... ..........
EU255290        .......... .......... .......... .......... C......... ..........
OXA-113         .......... .......... .......... .......... C......... ..........
EU255291        .......... .......... .......... .......... C......... ..........
OXA-51          .......... .......... .......... .......... C......... ..........
OXA-132         .......... .......... .......... .......... C......... ..........
OXA-99          .......... .......... .......... .......... C......... ..........
OXA-64          .......... .......... .......... .......... C......... ..........
OXA-111         .......... .......... .......... .......... C......... ..........
OXA-106         .......... .......... .......... .......... .......... ..........
OXA-70          .......... .......... .......... .......... .......... .C........
EU255294        .......... .......... .......... .......... .......... .C........
OXA-148         .......... .......... .......... .......... .......... ..........
EU255292        .......... .......... .......... .......... C......... ..........
OXA-86          .......... .......... .......... .......... T......... ..........
OXA-87          .......... .......... .......... .......... T.....C... ..........
OXA-67          .......... .......... .......... .......... T......... ..........
OXA-100         .......... .......... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   605        615        625        635        645        655
OXA-65          CAATCCATGC TATTCATAGA AGAAAAGAAT GGAAACAAAA TATACGCAAA AAGTGGTTGG
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-66          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-90          .......... .......... .......... .......... .......... ..........
OXA-130         .......... .......... .......... .....T.... .......... ..........
EU255295        .......... .......... .......... .....T.... .......... ..........
OXA-108         .......... .......... .......... .....T.... .......... ..........
OXA-95          .......... .......... .......... .....T.... .......... ..........
OXA-88          .......... .......... .......... .....T.... .......... ..........
OXA-94          .......... .......... .......... .....T.... .......... ..........
OXA-104         .........T .......... .......... .....T.... .......... ..........
OXA-107         .......... .......... .......... .....T.... .......... ..........
OXA-69          .......... .......... .......... .....T.... .......... ..........
OXA-110         .......... .......... .......... .....T.... .......... ..........
OXA-112         .......... .......... .......... .....T.... .......... ..........
OXA-92          .......... .......... .......... .....T.... .......... ..........
EU255289        .......... .......... .......... .....T.... .......... ..........
EU255293        .........T .......... .......... .....T.... .......... ..........
OXA-89          .........T .......... .......... .....T.... .......... ..........
OXA-149         .......... .......... .......... .....T.... .......... ..........
OXA-77          .......... .......... .......... .....T.... .......... ..........
OXA-128         .......... .......... .......... .....T.... .......... ..........
OXA-68          .......... .......... .......... .....T.... .......... ..........
OXA-144         ........T. .......... .......... .....T.... .......... ..........
OXA-117         .......... .......... .......... .....T.... .......... ..........
OXA-98          .......... .......... .......... .....T.... .......... ..........
OXA-78          .......... .......... .......... .....T.... .......... ..........
OXA-91          .......... .......... .......... .....T.... .......... ..........
OXA-116         .........T .......... .......... .....T.... .......... ..........
OXA-150         .......... .G........ .......... .....T.... .......... ..........
OXA-75          .......... .......... .......... .....T.... .......... ..........
OXA-93          .......... .......... .......... .....T.... .......... ..........
OXA-71          .......... .......... .......... .....T.... .......... ..........
EU255290        .......... .......... .......... .....T.... .......... ..........
OXA-113         .......... .......... .......... .....T.... .......... ..........
EU255291        .........T .......... .......... .....T.... .......... ..........
OXA-51          .........T .......... .......... .....T.... .......... ..........
OXA-132         .........T .......... .......... .....T.... .......... ..........
OXA-99          .........T .......... .....T.... .....T.... .......... ..........
OXA-64          .........T .......... .......... .....T.... .......... ..........
OXA-111         .........T .......... .....T.... .....T.... .......... ..........
OXA-106         .......... .......... .......... .....T.... .......... ..........
OXA-70          .......... .......... .......... .....T.... .......... ..........
EU255294        .......... .......... .......... .....T.... .......... ..........
OXA-148         A......... .......... .......... .....T.... .......... ..........
EU255292        .........T .......... .......... .....T.... .......... ..........
OXA-86          .........T .......... .......... .....T.... .......... ..........
OXA-87          .........T .......... .......... .....T.... .......... ..........
OXA-67          .........T .......... .......... .....T.... .......... ..........
OXA-100         .......... .......... .......... .....T.... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   665        675        685        695        705        715
OXA-65          GGATGGGATG TAAACCCACA AGTAGGCTGG TTAACTGGAT GGGTTGTTCA GCCTCAAGGG
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-66          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-79          ...G...... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-131         .......... ......A... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-90          .......... ..G....... .......... .......... .......... .........A
OXA-130         .......... ..G....... .......... .......... .......... ..........
EU255295        .......... ..G....... .......... .......... .......... ..........
OXA-108         .......... ..G....... .......... .......... .......... ..........
OXA-95          .......... ..G....... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-94          .......... .......... .......... .......... .......... ......T...
OXA-104         .......... ..G....... .......... .......... .......... .........A
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-69          .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........
OXA-92          .......... .......... .......... .......... C......... ..........
EU255289        .......... .......... .......... .......... .......... ..........
EU255293        .......... ..G....... .......... .......... .......... ..........
OXA-89          .......... .......... .......... .......... .......... ..........
OXA-149         .......... .......... .......... .......... .......... .........A
OXA-77          .......... .......... .......... .......... .......... ..........
OXA-128         .......... .......... .......... .......... .......... ..........
OXA-68          .......... .......... .......... .......... .......... ..........
OXA-144         .......... .......... .......... .......... .......... ..........
OXA-117         .......... .......... .......... .......A.. .......... ..........
OXA-98          .......... .......... .......... .......... .......... ..........
OXA-78          .......... .......... .......... .......... .......... ..........
OXA-91          .......... ..G....... .......... .......... .......... ..........
OXA-116         .......... ..G....... ...G..T... .......... .......... ..........
OXA-150         .......... .......... .......... .......... .......... ..........
OXA-75          .......... .......... .......... .......... .......... ..........
OXA-93          .......... .......... .......... .......... .......... ..........
OXA-71          .......... ..G....... .......... .......... .......... .........A
EU255290        .......... ..G....... .......... .......... .......... .........A
OXA-113         .......... ..G....... .......... .......... .......... .........A
EU255291        .......... ..G....... .......... .......... .......... .........A
OXA-51          .......... ..G....... .......... .......... .......... .........A
OXA-132         .......... ..G....... .......... .......... .......... .........A
OXA-99          .......... ..G....... .......... .......... .......... .........A
OXA-64          .......... ..G....... .......... .......... .......... ..........
OXA-111         .......... ..G....... .......... .......... .......... .........A
OXA-106         .......... ..G....... .......... .......... .......... ..........
OXA-70          .......... ..G....... .......... .......... .......... ..........
EU255294        .......... ..G....... .......... .......... .........G ..........
OXA-148         .......... ..G....... .......... .......... .......... ..........
EU255292        .......... ..G....... .......... .......... .......... .........A
OXA-86          .......... ..G....... .......... .......... .......... .........A
OXA-87          .......... ..G....... .......... .......... .......... .........A
OXA-67          .......... ..G....... .......... .......... .......... .........A
OXA-100         .......... ..G....... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   725        735        745        755        765        775
OXA-65          AATATTGTAG CGTTCTCCCT TAACTTAGAA ATGAAAAAAG GAATACCTAG CTCTGTTCGA
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-66          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-90          .......... .......... .......... .......... .......... ..........
OXA-130         .......... .......... .......... .......... .......... ..........
EU255295        .......... .......... .......... .......... .......... ..........
OXA-108         .......... .......... .......... .......... .......... ..........
OXA-95          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-94          .......... .......... .......... .......... .......... ..........
OXA-104         ..........A..........  .......... .......... .......... ..........
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-69          .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .....C.... .......... .......... .......... .......... ..........
OXA-92          .......... .......... .......... .......... .......... ..........
EU255289        .......... .......... .......... .......... .......... ..........
EU255293        .......... .......... .......... .......... .......... ..........
OXA-89          .......... .......... .......... .......... .......... ..........
OXA-149         .......... .......... .......... .......... .......... ..........
OXA-77          .......... .......... .......... .......... .......... ..........
OXA-128         .......... .......... .......... .......... .......... ..........
OXA-68          .......... .......... .......... .......... .......... ..........
OXA-144         .......... .......... .......... .......... .......... ..........
OXA-117         .......... .......... .......... .......... .......... ..........
OXA-98          .......... .......... .......... .......... .......... ..........
OXA-78          .......... .......... .......... .......... ...C...... ..........
OXA-91          .......... .......... .......... .......... .......... ..........
OXA-116         .......... .......... .......... .......... .......... ..........
OXA-150         .......... .......... .......... .......... .......... ..........
OXA-75          .......... .......... .......... .......... .....T.... ..........
OXA-93          .......... .......... .......... .......... .......... ..........
OXA-71          ..........A..........  .......... .......... .......... ..........
EU255290        ..........A..........  .......... .......... .......... ..........
OXA-113         .......... .......... .......... .......... .......... ..........
EU255291        .......... .......... .......... .......... .......... ..........
OXA-51          .......... .......... .......... .......... .......... ..........
OXA-132         .......... .......... .......... .......... .......... ..........
OXA-99          .......... .......... .......... .......... .......... ..........
OXA-64          .......... .......... .......... .......... .......... ..........
OXA-111         .......... .......... .......... .......... .......... ..........
OXA-106         .......... .......... .......... .......... .......... ..........
OXA-70          .......... .......... .......... .......... .......... ..........
EU255294        .......... .......... .......... .......... .....G.... ..........
OXA-148         .......... .......... .......... .......... .......... ..........
EU255292        .......... .......... .......... .......... .......... ..........
OXA-86          .......... .......... .......... .......... .......... ..........
OXA-87          .......... .......... .......... .......... .......... ..........
OXA-67          .......... .......... .......... .......... .......... ..........
OXA-100         .......... .......... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|
                   785        795        805        815        825
OXA-65          AAAGAGATTA CTTATAAAAG TTTAGAACAA TTAGGTATTT TATAG
OXA-82          .......... .......... C......... .......... .....
OXA-138         .......... .......... C......... .......... .....
OXA-115         .......... .......... C......... .......... .....
OXA-109         .......... .......... C......... .......... .....
OXA-80          .......... .......... C......... .......... .....
OXA-66          .......... .......... C......... .......... .....
OXA-83          .......... .......... C......... .......... .....
EU255296        .......... .......... C......... .......... .....
OXA-79          .......... .......... C......... .......... .....
OXA-76          .......... .......... C......A.. .......... .....
OXA-131         .......... .......... C......... .......... .....
OXA-84          .......... .......... C......... .......... .....
OXA-90          .......... .......... .......... .......... .....
OXA-130         .......... .......... .......... .......... .....
EU255295        .......... .......... .......... .......... .....
OXA-108         .......... .......... .......... .......... .....
OXA-95          .......... .......... .......... .......... .....
OXA-88          .......... .......... .......... .......... .....
OXA-94          .......... .......... .......... .......... .....
OXA-104         .......... .......... .......... .......... .....
OXA-107         .......... .......... .......... .......... .....
OXA-69          .......... .......... .......... .......... .....
OXA-110         .......... .......... .......... .......... .....
OXA-112         .......... .......... .......... .......... .....
OXA-92          .......... .......... .......... .......... .....
EU255289        .......... .......... .......... .......... .....
EU255293        .......... .......... .......... .......... .....
OXA-89          .......... .......... .......... .......... .....
OXA-149         .......... .......... .......... .......... .....
OXA-77          .......... .......... C......... .......... .....
OXA-128         .......... .......... C......... .......... .....
OXA-68          .......... .......... C......... .......... .....
OXA-144         .......... .......... C......... .......... .....
OXA-117         .......... .......... C......... .......... .....
OXA-98          .......... .......... C......... .......... .....
OXA-78          .......... .......... C......... .......... .....
OXA-91          .......... .......... .......... .......... .....
OXA-116         .......... .......... ....------ ---------- -----
OXA-150         .......... .......... C......... .......... .....
OXA-75          .......... ......G.G. .......... .......... .....
OXA-93          .......... .......... C......... .......... .....
OXA-71          .......... .......... .......... .......... .....
EU255290        .......... .......... .......... .......... .....
OXA-113         .......... .......... C......... .......... .....
EU255291        .........G .......... .......... .......... .....
OXA-51          .......... .......... .......... .......... .....
OXA-132         ........G. .......... .......... .......... .....
OXA-99          .......... .......... .......... .......... .....
OXA-64          .......... .......... .......... .......... .....
OXA-111         .......... .......... .......... .......... .....
OXA-106         .......... .......... .......... .......... .....
OXA-70          .......... .......... .......... .......... .....
EU255294        .......... .......... .......... .......... .....
OXA-148         .......... .......... .......... .......... .....
EU255292        .......... .......... .......... .......... .....
OXA-86          .......... .......... .......... .......... .....
OXA-87          .......... .......... .......... .......... .....
OXA-67          .......... .......... .......... .......... .....
OXA-100         .......... .......... .......... .......... .....
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2. Amino-acid alignment for all 60 OXA-51-like enzymes. Substitutions are labelled with respect to OXA-65.

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     5         15         25         35         45         55
OXA-65          MNIKALLLIT SAIFISACSP YIVTANPNHS ASKSDEKAEK IKNLFNEAHT TGVLVIQQGQ
OXA-82          .......... .......... .......... .....V.... .......... ..........
OXA-138         ....T..... .......... .......... .....V.... .......... ..........
OXA-115         .......... .......... .......... .....V.... .......... ..........
OXA-109         .......... .......... .......... .....V.... .......... ..........
OXA-80          .......... .......... .......... .....V.... .......... ..........
OXA-66          .......... .......... .......... .....V.... .......... ..........
OXA-83          .......... .......... .......... .....V.... .......... ..........
EU255296        .......... .......... .......... .....V.... .......... ..........
OXA-79          .......... .......... .......... .....V.... .......... ..........
OXA-76          .......... .......... .......... .....V.... .......... ..........
OXA-131         .......... .......... .......... .....V.... .......... ..........
OXA-84          .......... .......... .......... .....V.... .......... ..........
OXA-90          .......... .......... .......... .......... .......... ..........
OXA-130         .......... .......... ....T..... .......... .......... ..........
EU255295        .......... .......... ....T..... .......... .......... ..........
OXA-108         .......... .......... ....T..... .......... .......... ..........
OXA-95          ......F... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .....K.... .......... ..........
OXA-94          ...Q...... .......... ...S...... .......... .......... ..........
OXA-104         .......... .......... .......... .......... .......... ..........
OXA-107         .......... .......... .......... .....D.... .......... ......H...
OXA-69          .......... .......... .......... .....D.... .......... ......H...
OXA-110         .......... .......... .......... .....D.... .......... ......H...
OXA-112         .......... .......... .......... .....D.... .......... ......H...
OXA-92          .......... .......... .......... .....D.... .......... ......H...
EU255289        .......... .......... .......... .....D.... .......... ......H...
EU255293        .......... .......... .......... .....D.... .......... ......H...
OXA-89          ....T..... .........H ...S...... .......... .......... ..........
OXA-149         .......... .......... ...S...... .......... .......... ..........
OXA-77          .......... .......... ...S...... .......... .......... ..........
OXA-128         .......... .......... ...S...... .......... .......... ..........
OXA-68          .......... .......... ...S...... .......... .......... ..........
OXA-144         .......... .......... ...S...... .......... .......... ..........
OXA-117         .......... .......... ...S...... .......... .......... ..........
OXA-98          .......... .......... ...S...... .......... .......... ..........
OXA-78          .......... .......... ...S...... .......... .......... ..........
OXA-91          .......... .......... ...S...... .......... .......... ..........
OXA-116         ------.... .......... ...S...... .....D.... .......... ..........
OXA-150         .......... .......... ...S...... .......... .......... ..........
OXA-75          ...Q...... .......... ........Y. .......... .......... ..........
OXA-93          .......... .T........ .......... .......... .......... ..........
OXA-71          .......... .......... .......... .......... .......... ..........
EU255290        .......... N......... .......... .......... .......... ..........
OXA-113         .......... .......... .......... .......... .......... ..........
EU255291        .......... .......... .......... .......... .......V.. ..........
OXA-51          ....T..... .......... .......... .......... .......V.. ..........
OXA-132         ....T..... .......... .......... .......... .......V.. ..........
OXA-99          ....T..... .......... .......... .......... .......V.. ......R...
OXA-64          .......... .......... .......... .......G.. .......... ..........
OXA-111         ....T..... .......... ...S...... .......... .......... ..........
OXA-106         .......... .......... .......... .....K.... .......... ..........
OXA-70          .......... .......... .......... .....K.... .......... ..........
EU255294        .......... .......... .......... .....K.... .......... ..........
OXA-148         .......... .......... .......... .....K.... .......... ..........
EU255292        .......... .......... .......... .....K.... .......... ..........
OXA-86          ....T..... .T........ .......... T......... .......... ..........
OXA-87          ....T..... .T........ .......... T......... .......... ..........
OXA-67          .......... .T........ .......... T......... .......... ..........
OXA-100         .......... .T........ .......... T......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    65         75         85         95        105        115
OXA-65          TQQSYGNDLA RASTEYVPAS TFKMLNALIG LEHHKATTTE VFKWDGKKRL FPEWEKDMTL
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-66          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-90          .......... .......... .......... .......... .......... ..........
OXA-130         .......... .......... .......... .......... .......... ..........
EU255295        .......... .......... .......... .......... .......... ..........
OXA-108         .......... .......... .......... .......... .......... ..........
OXA-95          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-94          .......... .......... .......... .......... .......... ..........
OXA-104         .......... .......... .......... .......... .......... ......N...
OXA-107         .......... .......... .......... .......... ......E... ......N...
OXA-69          .......... .......... .......... .......... ......E... ......N...
OXA-110         .......... .......... .......... .......... ......E... ......N...
OXA-112         .......... .......... .......... .......... ......E... ......N...
OXA-92          .......... .......... .......... .......... ......E... ......N...
EU255289        .......... .......... .......... .......... I.....Q... ..........
EU255293        .......... .......... .......... .......... ....N.Q... ..........
OXA-89          .......... .......... .......... .......... ......Q... ......N...
OXA-149         .......... .......... .......... .......... ......Q... ......N...
OXA-77          .......... .......... .......... .......... ......Q... ......N...
OXA-128         .......V.. .......... .......... .......... ......Q... ......N...
OXA-68          .......... .......... .......... .......... ......Q... ......N...
OXA-144         .......... .......... .......... .......... ......Q... ......N...
OXA-117         .......... .......... .......... .......... ......Q... ......N...
OXA-98          .......... .......... .......... .......... ......Q... ......N...
OXA-78          .......... .......... .......... .........A ......Q... ......N...
OXA-91          .......... .......... .......... .......... ......Q... ......N...
OXA-116         .......... .......... .......... .......... ......Q... ......N...
OXA-150         .......... .......... .......... .......... ......Q... ......N...
OXA-75          .......... .......... .......... .......... ......Q... ......N...
OXA-93          .......... .......... .........S .......... ......Q... ..........
OXA-71          .......... .......... .......... .....T.... ......Q... ..........
EU255290        .......... .......... .......... .....T.... ......Q... C.........
OXA-113         .......... .......... .......... .....T.... ......Q... ..........
EU255291        .......... .......... .......... .......... ......Q... ..........
OXA-51          .......... .......... .......... .......... ......Q... ..........
OXA-132         .......... .......... .......... .......... ......Q... ..........
OXA-99          .......... .......... .......... .......... ......Q... ..........
OXA-64          .......... .......... .......... .......... ......Q... ..........
OXA-111         .......... .......... .......... .......... ......Q... ..........
OXA-106         .......... .......... .......... .......... ....N.Q... ..........
OXA-70          .......... .......... .......... .......... ....N.Q... ..........
EU255294        .......... .......... .......... .......... ....N.Q... ..........
OXA-148         .......... .......... .......... ......S... ....N.Q... ..........
EU255292        .......... .......... .......... .......... ....N.Q... ..........
OXA-86          .......... ...I...... .......... .......... I.....Q... ..........
OXA-87          .......... ...I...... .......... .......... I.....Q... ..........
OXA-67          .......... .......... .......... .......... I.....Q... ..........
OXA-100         .......... .......... .......... .......... I.....Q... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   125        135        145        155        165        175
OXA-65          GDAMKASAIP VYQDLARRIG LELMSKEVKR VGYGNADIGT QVDNFWLVGP LKITPQQEAQ
OXA-82          .......... .......... .......... .......... ......V... ..........
OXA-138         .......... .......... .......... .......... ......V... ..........
OXA-115         .........L .......... .......... .......... ......V... ..........
OXA-109         .........Q .......... .......... .......... .......... ..........
OXA-80          .........L .......... .......... .......... .......... ..........
OXA-66          .......... .......... .......... .......... .......... ..........
OXA-83          ........L. .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... ......Q... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-131         ........V. .......... .......... .......... .......... ..........
OXA-84          .........S .......... .......... .......... .......A.. ..........
OXA-90          .......... .......... .......... .......... .......... ..........
OXA-130         .......... .......... .......... .....T.... .......... ..........
EU255295        .......... .......... .......... .....T.... .......M.. ..........
OXA-108         .......... .......... .......... .....T.... ......V... ..........
OXA-95          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-94          .......... .......... .......... .......... .......... ..........
OXA-104         .......... .......... .....N.... .......... .......... ..........
OXA-107         .......... .......... .......... .......... ......V... ..........
OXA-69          .......... .......... .......... .......... .......... ..........
OXA-110         ........L. .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........
OXA-92          .......... .......... .......... .......... .......... ..........
EU255289        .......... .......... .......... .......... .......... ..........
EU255293        .......... .......... .....N.... ......H... .......... ..........
OXA-89          .......... .......... .....N.... .......... .......... ..........
OXA-149         .......... .......... .....N...H .......... .......... ..........
OXA-77          .......... .....P.... .....N.... .......... .......... ..........
OXA-128         .......... .......... .....N.... .......... .......... ..........
OXA-68          .......... .......... .....N.... .......... .......... ..........
OXA-144         .......... .......... .....N.... .......... .......... ..........
OXA-117         .......... .......... .....N.... .......... .......... ..........
OXA-98          .......... .......... .....N.... .......... .......... ..........
OXA-78          .......... .......... .....N.... .......... .......... ..........
OXA-91          .......... .......... .....N.... .......... .......... ..........
OXA-116         .......... .......... .....N.... .......... .......... ..........
OXA-150         .......... .......... .....N...H .......... .......... ..........
OXA-75          .......... .......... .....N.... I......... .......... ..........
OXA-93          .......... .......... .....N.... .......... .......... ..........
OXA-71          .......... .......... .......... .......... .......... ..........
EU255290        .......... .......... .......... .......... .......... ..........
OXA-113         .......... .......... .......... .......... ......V... ..........
EU255291        .......... .......... .......... .......... .......... ..........
OXA-51          .......... .......... .......... .......... .......... ..........
OXA-132         .......... .......... .......... .......... .......... ..........
OXA-99          .......... .......... .......... .......... .......... ..........
OXA-64          .......... .......... .......... .......... .......... ..........
OXA-111         .......... .......... .......... .......... .......... ..........
OXA-106         .......... .......... .....N.... .......... .......... ..........
OXA-70          .......... .......... .....N.... .......... .......... ..........
EU255294        .......... .......... .....N.... .......... .......... ..........
OXA-148         .......... .......... .....N.... .......... .......... ..........
EU255292        .......... .......... .....N.... .......... .......... ..........
OXA-86          .......... .......... .......... .......... .......... ..........
OXA-87          .......... .......... .......... .......... .......... ..........
OXA-67          .......... .......... .......... .......... .......... ..........
OXA-100         .......... .......... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   185        195        205        215        225        235
OXA-65          FAYKLANKTL PFSQKVQDEV QSMLFIEEKN GNKIYAKSGW GWDVNPQVGW LTGWVVQPQG
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-66          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... ..........G..........  ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .....Q.... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-90          .......... .......... .......... .......... ....D..... ..........
OXA-130         .......... .......... .......... .......... ....D..... ..........
EU255295        .......... .......... .......... .......... ....D..... ..........
OXA-108         .......... .......... .......... .......... ....D..... ..........
OXA-95          .......... .......... .......... .......... ....D..... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-94          .......... .......... .......... .......... .......... ........H.
OXA-104         .......... .......... .......... .......... ....D..... ..........
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-69          .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........
OXA-92          .......... .......... .......... .......... .......... ...S......
EU255289        .......... .......... .......... .......... .......... ..........
EU255293        .......... .......... .......... .......... ....D..... ..........
OXA-89          .T........ .......... .......... .......... .......... ..........
OXA-149         .......... ...P...... .......... .......... .......... ..........
OXA-77          .......... .......... .......... .......... .......... ..........
OXA-128         .......... ....E..... .......... .......... .......... ..........
OXA-68          .......... ....E..... .......... .......... .......... ..........
OXA-144         .......... ....E..... ..I....... .......... .......... ..........
OXA-117         .......... .......... .......... .......... .......... ..E.......
OXA-98          .......... .......... .......... .......... .......... ..........
OXA-78          .......... .......... .......... .......... .......... ..........
OXA-91          .......... .......... .......... .......... ....D..... ..........
OXA-116         .......... .......... .......... .......... ....D..... ..........
OXA-150         .......... .......... .......... .......... .......... ..........
OXA-75          .......... .......... .......... .......... .......... ..........
OXA-93          .......... .......... .......... .......... .......... ..........
OXA-71          .......... ...P...... .......... .......... ....D..... ..........
EU255290        .......... ...P...... .......... .......... ....D..... ..........
OXA-113         .......... ...P...... .......... .......... ....D..... ..........
EU255291        .......... ...P...... .......... .......... ....D..... ..........
OXA-51          .......... ...P...... .......... .......... ....D..... ..........
OXA-132         .......... ...P...... .......... .......... ....D..... ..........
OXA-99          .......... ...P...... ........M. .......... ....D..... ..........
OXA-64          .......... ...P...... .......... .......... ....D..... ..........
OXA-111         .......... ...P...... ........M. .......... ....D..... ..........
OXA-106         .......... .......... .......... .......... ....D..... ..........
OXA-70          .......... .......H.. .......... .......... ....D..... ..........
EU255294        .......... .......H.. .......... .......... ....D..... ......R...
OXA-148         .......... .......... K......... .......... ....D..... ..........
EU255292        .......... ...P...... .......... .......... ....D..... ..........
OXA-86          .......... ...L...... .......... .......... ....D..... ..........
OXA-87          .......... ...L.A.... .......... .......... ....D..... ..........
OXA-67          .......... ...L...... .......... .......... ....D..... ..........
OXA-100         .......... .......... .......... .......... ....D..... ..........
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                ....|....| ....|....| ....|....| ....|
                   245        255        265        275
OXA-65          NIVAFSLNLE MKKGIPSSVR KEITYKSLEQ LGIL*
OXA-82          .......... .......... .......... ....*
OXA-138         .......... .......... .......... ....*
OXA-115         .......... .......... .......... ....*
OXA-109         .......... .......... .......... ....*
OXA-80          .......... .......... .......... ....*
OXA-66          .......... .......... .......... ....*
OXA-83          .......... .......... .......... ....*
EU255296        .......... .......... .......... ....*
OXA-79          .......... .......... .......... ....*
OXA-76          .......... .......... .........K ....*
OXA-131         .......... .......... .......... ....*
OXA-84          .......... .......... .......... ....*
OXA-90          .......... .......... .......... ....*
OXA-130         .......... .......... .......... ....*
EU255295        .......... .......... .......... ....*
OXA-108         .......... .......... .......... ....*
OXA-95          .......... .......... .......... ....*
OXA-88          .......... .......... .......... ....*
OXA-94          .......... .......... .......... ....*
OXA-104         .......... .......... .......... ....*
OXA-107         .......... .......... .......... ....*
OXA-69          .......... .......... .......... ....*
OXA-110         .......... .......... .......... ....*
OXA-112         .....P.... .......... .......... ....*
OXA-92          .......... .......... .......... ....*
EU255289        .......... .......... .......... ....*
EU255293        .......... .......... .......... ....*
OXA-89          .......... .......... .......... ....*
OXA-149         .......... .......... .......... ....*
OXA-77          .......... .......... .......... ....*
OXA-128         .......... .......... .......... ....*
OXA-68          .......... .......... .......... ....*
OXA-144         .......... .......... .......... ....*
OXA-117         .......... .......... .......... ....*
OXA-98          .......... .......... .......... ....*
OXA-78          .......... ....T..... .......... ....*
OXA-91          .......... .......... .......... ....*
OXA-116         .......... .......... .........-- -----
OXA-150         .......... .......... .......... ....*
OXA-75          .......... .....S.... .....RG... ....*
OXA-93          .......... .......... .......... ....*
OXA-71          .......... .......... .......... ....*
EU255290        .......... .......... .......... ....*
OXA-113         .......... .......... .......... ....*
EU255291        .......... .......... ...A...... ....*
OXA-51          .......... .......... .......... ....*
OXA-132         .......... .......... ..M....... ....*
OXA-99          .......... .......... .......... ....*
OXA-64          .......... .......... .......... ....*
OXA-111         .......... .......... .......... ....*
OXA-106         .......... .......... .......... ....*
OXA-70          .......... .......... .......... ....*
EU255294        .......... .....A.... .......... ....*
OXA-148         .......... .......... .......... ....*
EU255292        .......... .......... .......... ....*
OXA-86          .......... .......... .......... ....*
OXA-87          .......... .......... .......... ....*
OXA-67          .......... .......... .......... ....*
OXA-100         .......... .......... .......... ....*
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3. Nucleotide alignment of *bla*$_{\text{OXA-66-like}}$ genes. Substitutions are labelled with respect to *bla*$_{\text{OXA-66}}$.

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    5          15         25         35         45         55
OXA-66          atgaacatta aagcactctt acttataaca agcgctattt ttatttcagc ctgctcacct
OXA-65          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-138         .......... ..a.c..... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    65         75         85         95        105        115
OXA-66          tatatagtga ctgctaatcc aaatcacagc gcttcaaaat ctgatgtaaa agcagagaaa
OXA-65          .......... .......... .........t .......... ......a... ..........
OXA-88          .......... .......... .......... .......... .....aa... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   125        135        145        155        165        175
OXA-66          attaaaaatt tatttaacga agcacacact acgggtgttt tagttatcca acaaggccaa
OXA-65          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   185        195        205        215        225        235
OXA-66          actcaacaaa gctatggtaa tgatcttgct cgtgcttcga ccgagtatgt acctgcttcg
OXA-65          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   245        255        265        275        285        295
OXA-66          accttcaaaa tgcttaatgc tttgatcggc cttgagcacc ataaggcaac caccacagaa
OXA-65          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   305        315        325        335        345        355
OXA-66          gtatttaagt gggatggtaa aaaaaggtta ttcccagaat gggaaaagga catgacccta
OXA-65          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   365        375        385        395        405        415
OXA-66          ggcgatgcca tgaaagcttc cgctattcca gtttatcaag atttagctcg tcgtattgga
OXA-65          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .........g .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... ....c..... .......... .......... ..........
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... ........T. .......... .......... ..........
OXA-109         .......... .......... ........A. .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... ........t. .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......t.. .......... .......... ..........
OXA-131         .......... .......... ....g..... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   425        435        445        455        465        475
OXA-66          cttgagctca tgtctaagga agtgaagcgt gttggttatg gcaatgcaga tatcggtacc
OXA-65          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
OXA-82          .......... ....A..... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   485        495        505        515        525        535
OXA-66          caagtcgata atttttggct ggtgggtcct ttaaaaatta ctcctcagca agaggcacag
OXA-65          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
OXA-82          .......... ........G. .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
EU255296        .......... .........a .......... .......... .......... ..........
OXA-115         .......... ........g. .......... .......... .......... ..........
OXA-138         .......... ........g. .......... .......... .......... ..........
OXA-84          .......... .......... ..c....... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   545        555        565        575        585        595
OXA-66          tttgcttaca agctagctaa taaaacgctt ccatttagcc aaaaagtcca agatgaagtg
OXA-65          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   605        615        625        635        645        655
OXA-66          caatccatgc tattcataga agaaaagaat ggaaacaaaa tatacgcaaa aagtggttgg
OXA-65          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .....t.... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   665        675        685        695        705        715
OXA-66          ggatgggatg taaacccaca agtaggctgg ttaactggat gggttgttca gcctcaaggg
OXA-65          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-79          ...G...... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-131         .......... ......a... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   725        735        745        755        765        775
OXA-66          aatattgtag cgttctccct taacttagaa atgaaaaaag gaatacctag ctctgttcga
OXA-65          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|
                   785        795        805        815        825
OXA-66          aaagagatta cttataaaag cttagaacaa ttaggtattt tatag
OXA-65          .......... .......... t......... .......... .....
OXA-88          .......... .......... t......... .......... .....
OXA-76          .......... .......... .......a.. .......... .....
OXA-79          .......... .......... .......... .......... .....
OXA-83          .......... .......... .......... .......... .....
OXA-82          .......... .......... .......... .......... .....
OXA-80          .......... .......... .......... .......... .....
OXA-109         .......... .......... .......... .......... .....
EU255296        .......... .......... .......... .......... .....
OXA-115         .......... .......... .......... .......... .....
OXA-138         .......... .......... .......... .......... .....
OXA-84          .......... .......... .......... .......... .....
OXA-131         .......... .......... .......... .......... .....
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4. Amino-acid alignment of OXA-66-like enzymes. Substitutions are labelled with respect to OXA-66.

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    5         15         25         35         45         55
OXA-66          MNIKALLLIT SAIFISACSP YIVTANPNHS ASKSDVKAEK IKNLFNEAHT TGVLVIQQGQ
OXA-65          .......... .......... .......... .....E.... .......... ..........
OXA-88          .......... .......... .......... .....K.... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-138         ....T..... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    65         75         85         95        105        115
OXA-66          TQQSYGNDLA RASTEYVPAS TFKMLNALIG LEHHKATTTE VFKWDGKKRL FPEWEKDMTL
OXA-65          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-83          .......... .......... .......... .......... .......... ..........
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   125        135        145        155        165        175
OXA-66          GDAMKASAIP VYQDLARRIG LELMSKEVKR VGYGNADIGT QVDNFWLVGP LKITPQQEAQ
OXA-65          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .......... ..........
OXA-83          ........L. .......... .......... .......... .......... ..........
OXA-82          .......... .......... .......... .......... .....V.... ..........
OXA-80          .........L .......... .......... .......... .......... ..........
OXA-109         .........Q .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .....Q.... ..........
OXA-115         .........L .......... .......... .......... .....V.... ..........
OXA-138         .......... .......... .......... .......... .....V.... ..........
OXA-84          .........S .......... .......... .......... .......A.. ..........
OXA-131         ........V. .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   185        195        205        215        225        235
OXA-66          FAYKLANKTL PFSQKVQDEV QSMLFIEEKN GNKIYAKSGW GWDVNPQVGW LTGWVVQPQG
OXA-65          .......... .......... .......... .......... .......... ..........
OXA-88          .......... .......... .......... .......... .......... ..........
OXA-76          .......... .......... .......... .......... .......... ..........
OXA-79          .......... .......... .......... .......... .G........ ..........
OXA-83          .......... .......... .......... .......... .......... ..........
OXA-82          .......... .......... .......... .......... .......... ..........
OXA-80          .......... .......... .......... .......... .......... ..........
OXA-109         .......... .......... .......... .......... .......... ..........
EU255296        .......... .......... .......... .......... .......... ..........
OXA-115         .......... .......... .......... .......... .......... ..........
OXA-138         .......... .......... .......... .......... .......... ..........
OXA-84          .......... .......... .......... .......... .......... ..........
OXA-131         .......... .......... .......... .......... .....Q.... ..........
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              ....|....| ....|....| ....|....| ....
                 245        255        265
OXA-66        NIVAFSLNLE MKKGIPSSVR KEITYKSLEQ LGIL
OXA-65        .......... .......... .......... ....
OXA-88        .......... .......... .......... ....
OXA-76        .......... .......... .........K ....
OXA-79        .......... .......... .......... ....
OXA-83        .......... .......... .......... ....
OXA-82        .......... .......... .......... ....
OXA-80        .......... .......... .......... ....
OXA-109       .......... .......... .......... ....
EU255296      .......... .......... .......... ....
OXA-115       .......... .......... .......... ....
OXA-138       .......... .......... .......... ....
OXA-84        .......... .......... .......... ....
OXA-131       .......... .......... .......... ....
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5. Nucleotide alignment of *bla*$_{\text{OXA-69-like}}$ genes. Substitutions are labelled with respect to *bla*$_{\text{OXA-69}}$.

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    5         15         25         35         45         55
OXA-69          atgaacatta aagcactctt acttataaca agcgctattt ttatttcagc ctgctcacct
OXA-92          .......... .......... .......... .......... .......... ..........
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    65         75         85         95        105        115
OXA-69          tatatagtga ctgctaatcc aaatcacagt gcttcaaaat ctgatgacaa agcagagaaa
OXA-92          .......... .......... .......... .......... .......... ..........
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   125        135        145        155        165        175
OXA-69          attaaaaatt tatttaacga agcacacact acgggtgttt tagttatcca tcaaggtcaa
OXA-92          .......... .......... .......... .......... .......... ..........
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   185        195        205        215        225        235
OXA-69          actcaacaaa gctatggtaa tgatcttgct cgtgcttcga ccgagtatgt acctgcttcg
OXA-92          .......... .......... .......... .......... .......... ..........
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   245        255        265        275        285        295
OXA-69          accttcaaaa tgcttaatgc tttgatcggc cttgagcacc ataaggcaac caccacagaa
OXA-92          .......... .......... .......... .......... .......... ..........
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   305        315        325        335        345        355
OXA-69          gtatttaaat gggatgggga aaaaaggcta ttcccagaat gggaaaagaa catgacccta
OXA-92          .......... .......... .......... .......... .......... ..........
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   365        375        385        395        405        415
OXA-69          ggcgatgcta tgaaagcttc cgctattccg gtttatcaag atttagctcg tcgtattgga
OXA-92          .......... .......... .......... .......... .......... ..........
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... ....C..... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   425        435        445        455        465        475
OXA-69          cttgagctca tgtctaagga agtgaagcgt gttggttatg gcaatgcaga tatcggtacc
OXA-92          .......... .......... .......... .......... .......... ..........
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   485        495        505        515        525        535
OXA-69          caagtcgata atttttggct ggtgggtcct ctaaaaatta ctcctcagca agaggcacag
OXA-92          .......... .......... .......... .......... .......... ..........
OXA-107         .......... ........G. .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   545        555        565        575        585        595
OXA-69          tttgcttaca agctagctaa taaaacgctt ccatttagcc aaaaagtcca agatgaagtg
OXA-92          .......... .......... .......... .......... .......... ..........
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   605        615        625        635        645        655
OXA-69          caatccatgc tattcataga agaaaagaat ggaaataaaa tatacgcaaa aagtggttgg
OXA-92          .......... .......... .......... .......... .......... ..........
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   665        675        685        695        705        715
OXA-69          ggatgggatg taaacccaca agtaggctgg ttaactggat gggttgttca gcctcaaggg
OXA-92          .......... .......... .......... .......... c......... ..........
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   725        735        745        755        765        775
OXA-69          aatattgtag cgttctccct taacttagaa atgaaaaaag gaatacctag ctctgttcga
OXA-92          .......... .......... .......... .......... .......... ..........
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .....C.... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|
                   785        795        805        815        825
OXA-69          aaagagatta cttataaaag tttagaacaa ttaggtattt tatag
OXA-92          .......... .......... .......... .......... .....
OXA-107         .......... .......... .......... .......... .....
OXA-110         .......... .......... .......... .......... .....
OXA-112         .......... .......... .......... .......... .....
```

6. Amino-acid alignment of OXA-69-like enzymes. Substitutions are labelled with respect to OXA-69.

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    5         15         25         35         45         55
OXA-69          MNIKALLLIT SAIFISACSP YIVTANPNHS ASKSDDKAEK IKNLFNEAHT TGVLVIHQGQ
OXA-92          .......... .......... .......... .......... .......... ..........
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........

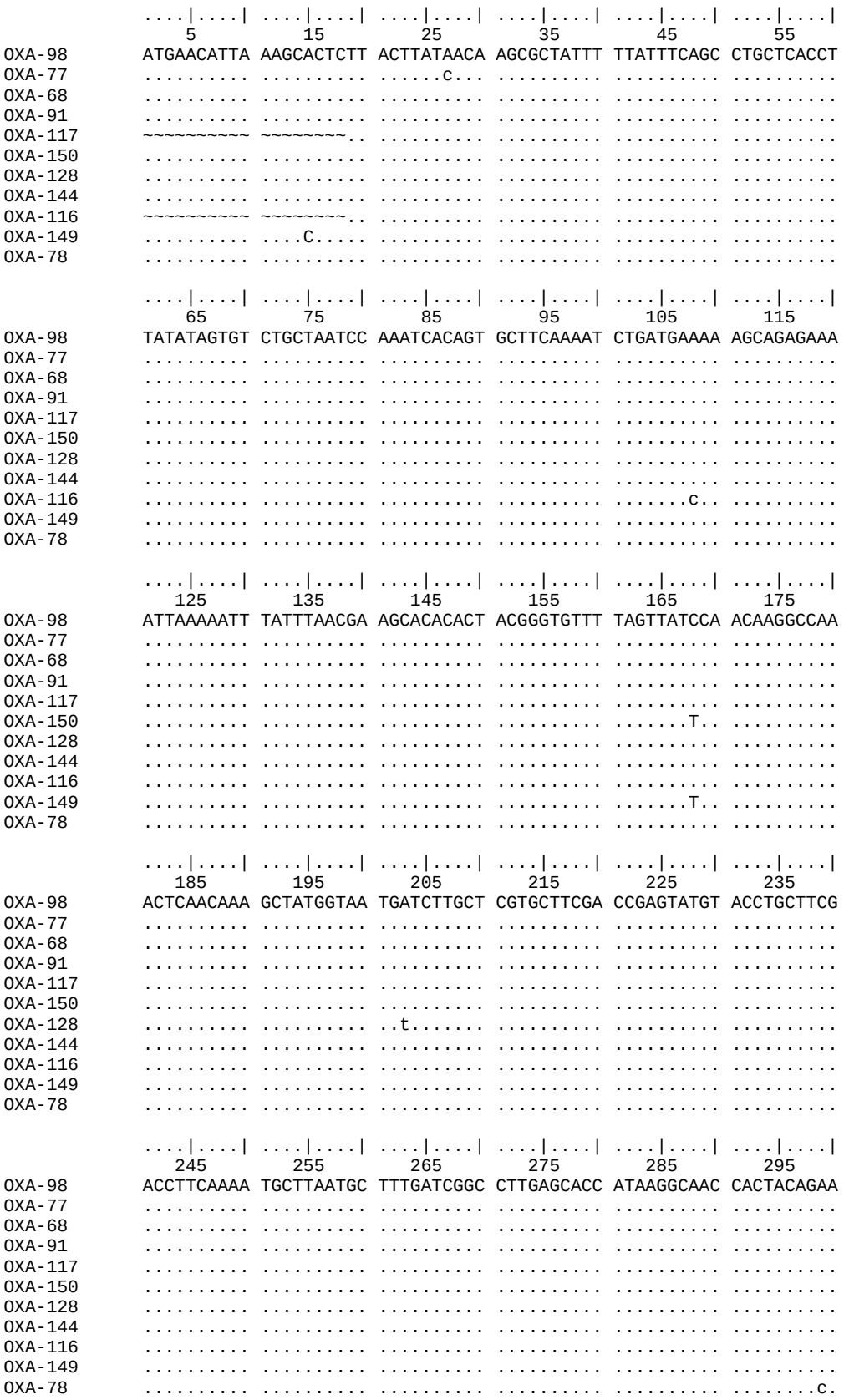
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    65         75         85         95        105        115
OXA-69          TQQSYGNDLA RASTEYVPAS TFKMLNALIG LEHHKATTTE VFKWDGEKRL FPEWEKNMTL
OXA-92          .......... .......... .......... .......... .......... ..........
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   125        135        145        155        165        175
OXA-69          GDAMKASAIP VYQDLARRIG LELMSKEVKR VGYGNADIGT QVDNFWLVGP LKITPQQEAQ
OXA-92          .......... .......... .......... .......... .......... ..........
OXA-107         .......... .......... .......... .......... ......V... ..........
OXA-110         ........L. .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   185        195        205        215        225        235
OXA-69          FAYKLANKTL PFSQKVQDEV QSMLFIEEKN GNKIYAKSGW GWDVNPQVGW LTGWVVQPQG
OXA-92          .......... .......... .......... .......... .......... ...S......
OXA-107         .......... .......... .......... .......... .......... ..........
OXA-110         .......... .......... .......... .......... .......... ..........
OXA-112         .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|
                   245        255        265        275
OXA-69          NIVAFSLNLE MKKGIPSSVR KEITYKSLEQ LGIL*
OXA-92          .......... .......... .......... ....*
OXA-107         .......... .......... .......... ....*
OXA-110         .......... .......... .......... ....*
OXA-112         .....P.... .......... .......... ....*
```

7. Nucleotide alignment of *bla*$_{\text{OXA-98-like}}$ genes. Substitutions are labelled with respect to *bla*$_{\text{OXA-98}}$.



```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   305        315        325        335        345        355
OXA-98          GTATTTAAGT GGGACGGGCA AAAAAGGCTA TTCCCAGAAT GGGAAAAGAA CATGACCCTA
OXA-77          .......... .......... .......... .......... .......... ..........
OXA-68          .......... .......... .......... .......... .......... ..........
OXA-91          .......... .......... .......... .......... .......... ..........
OXA-117         .......... .......... .......... .......... .......... ..........
OXA-150         .......... .......... .......... .......... .......... ..........
OXA-128         .......... .......... .......... .......... .......... ..........
OXA-144         .......... .......... .......... .......... .......... ..........
OXA-116         .......... .......... .......... .......... .......... ..........
OXA-149         .......... .......... .......... .......... .......... ..........
OXA-78          .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   365        375        385        395        405        415
OXA-98          GGCGATGCTA TGAAAGCTTC CGCTATTCCG GTTTATCAAG ATTTAGCTCG TCGTATTGGA
OXA-77          .......... .......... .......... .......... .....c.... ..........
OXA-68          .......... .......... .......... .......... .......... ..........
OXA-91          .......... .......... .......... .......... .......... ..........
OXA-117         .......... .......... .......... .......... .......... ..........
OXA-150         .......... .......... .......... .......... .......... ..........
OXA-128         .......... .......... .......... .......... .......... ..........
OXA-144         .......... .......... .......... .......... .......... ..........
OXA-116         .......... .......... .......... .......... .......... ..........
OXA-149         .......... .......... .......... .......... .......... ..........
OXA-78          .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   425        435        445        455        465        475
OXA-98          CTTGAACTCA TGTCTAATGA AGTGAAGCGT GTTGGTTATG GCAATGCAGA TATCGGTACC
OXA-77          .......... .......... .......... .......... .......... ..........
OXA-68          .......... .......... .......... .......... .......... ..........
OXA-91          .......... .......... .......... .......... .......... ..........
OXA-117         .......... .......... .......... .......... .......... ..........
OXA-150         .......... .......... ........A. .......... .......... ..........
OXA-128         .......... .......... .......... .......... .......... ..........
OXA-144         .......... .......... .......... .......... .......... ..........
OXA-116         .......... .......... .......... .......... .......... ..........
OXA-149         .......... .......... ........A. .......... .......... ..........
OXA-78          .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   485        495        505        515        525        535
OXA-98          CAAGTCGATA ATTTTTGGCT GGTGGGTCCT TTAAAAATTA CTCCTCAGCA AGAGGCACAA
OXA-77          .......... .......... .......... .......... .......... ..........
OXA-68          .......... .......... a......... .......... .......... ..........
OXA-91          .......... .......... .......... .......... .......... ..........
OXA-117         .......... .......... a......... .......... .......... ..........
OXA-150         .......... .......... .......... .......... .......... ..........
OXA-128         .......... .......... a......... .......... .......... ..........
OXA-144         .......... .......... A......... .......... .......... ..........
OXA-116         .......... .......... .......... .......... .......... .........g
OXA-149         .......... .......... .......... .......... .......... ..........
OXA-78          .......... .......... a......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   545        555        565        575        585        595
OXA-98          TTTGCTTACA AGCTAGCTAA TAAAACGCTT CCATTTAGCC AAAAAGTCCA AGATGAAGTG
OXA-77          .......... .......... .......... .......... .......... ..........
OXA-68          .......... .......... .......... .......... ..g....... ..........
OXA-91          .......... .......... .......... .......... .......... ..........
OXA-117         .......... .......... .......... .......... .......... ..........
OXA-150         .......... .......... .......... .......... .......... ..........
OXA-128         .......... .......... .......... .......... ..g....... ..........
OXA-144         .......... .......... .......... .......... ..G....... ..........
OXA-116         .......... .......... .......... .......... .......... ..........
OXA-149         .......... .......... .......... .......... C......... ..........
OXA-78          .......... .......... .......... .......... .......... ..........
```

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   605        615        625        635        645        655
OXA-98          CAATCCATGC TATTCATAGA AGAAAAGAAT GGAAATAAAA TATACGCAAA AAGTGGTTGG
OXA-77          .......... .......... .......... .......... .......... ..........
OXA-68          .......... .......... .......... .......... .......... ..........
OXA-91          .......... .......... .......... .......... .......... ..........
OXA-117         .......... .......... .......... .......... .......... ..........
OXA-150         ..........G. ......... .......... .......... .......... ..........
OXA-128         .......... .......... .......... .......... .......... ..........
OXA-144         ........T. .......... .......... .......... .......... ..........
OXA-116         .........t .......... .......... .......... .......... ..........
OXA-149         .......... .......... .......... .......... .......... ..........
OXA-78          .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   665        675        685        695        705        715
OXA-98          GGATGGGATG TAAACCCACA AGTAGGCTGG TTAACTGGAT GGGTTGTTCA GCCTCAAGGG
OXA-77          .......... .......... .......... .......... .......... ..........
OXA-68          .......... .......... .......... .......... .......... ..........
OXA-91          .......... ..g....... .......... .......... .......... ..........
OXA-117         .......... .......... .......... .......a.. .......... ..........
OXA-150         .......... .......... .......... .......... .......... ..........
OXA-128         .......... .......... .......... .......... .......... ..........
OXA-144         .......... .......... .......... .......... .......... ..........
OXA-116         .......... ..g....... ...g..t... .......... .......... ..........
OXA-149         .......... .......... .......... .......... .......... .........A
OXA-78          .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   725        735        745        755        765        775
OXA-98          AATATTGTAG CGTTCTCCCT TAACTTAGAA ATGAAAAAAG GAATACCTAG CTCTGTTCGA
OXA-77          .......... .......... .......... .......... .......... ..........
OXA-68          .......... .......... .......... .......... .......... ..........
OXA-91          .......... .......... .......... .......... .......... ..........
OXA-117         .......... .......... .......... .......... .......... ..........
OXA-150         .......... .......... .......... .......... .......... ..........
OXA-128         .......... .......... .......... .......... .......... ..........
OXA-144         .......... .......... .......... .......... .......... ..........
OXA-116         .......... .......... .......... .......... .......... ..........
OXA-149         .......... .......... .......... .......... .......... ..........
OXA-78          .......... .......... .......... .......... ...c...... ..........

                ....|....| ....|....| ....|....| ....|....| ....|
                   785        795        805        815        825
OXA-98          AAAGAGATTA CTTATAAAAG CTTAGAACAA TTAGGTATTT TATAG
OXA-77          .......... .......... .......... .......... .....
OXA-68          .......... .......... .......... .......... .....
OXA-91          .......... .......... t......... .......... .....
OXA-117         .......... .......... t......... .......... .....
OXA-150         .......... .......... .......... .......... .....
OXA-128         .......... .......... .......... .......... .....
OXA-144         .......... .......... .......... .......... .....
OXA-116         .......... .......... t......... .......... .....
OXA-149         .......... .......... T......... .......... .....
OXA-78          .......... .......... .......... .......... .....
```

8. Amino-acid alignment of OXA-98-like enzymes. Substitutions are labelled with respect to OXA-98.

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    5         15         25         35         45         55
OXA-98          MNIKALLLIT SAIFISACSP YIVSANPNHS ASKSDEKAEK IKNLFNEAHT TGVLVIQQGQ
OXA-77          .......... .......... .......... .......... .......... ..........
OXA-68          .......... .......... .......... .......... .......... ..........
OXA-91          .......... .......... .......... .......... .......... ..........
OXA-117         ~~~~~~.... .......... .......... .......... .......... ..........
OXA-150         .......... .......... .......... .......... .......... ..........
OXA-128         .......... .......... .......... .......... .......... ..........
OXA-144         .......... .......... .......... .......... .......... ..........
OXA-116         ~~~~~~.... .......... .......... .....D.... .......... ..........
OXA-149         .......... .......... .......... .......... .......... ..........
OXA-78          .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    65         75         85         95        105        115
OXA-98          TQQSYGNDLA RASTEYVPAS TFKMLNALIG LEHHKATTTE VFKWDGQKRL FPEWEKNMTL
OXA-77          .......... .......... .......... .......... .......... ..........
OXA-68          .......... .......... .......... .......... .......... ..........
OXA-91          .......... .......... .......... .......... .......... ..........
OXA-117         .......... .......... .......... .......... .......... ..........
OXA-150         .......... .......... .......... .......... .......... ..........
OXA-128         .......V.. .......... .......... .......... .......... ..........
OXA-144         .......... .......... .......... .......... .......... ..........
OXA-116         .......... .......... .......... .......... .......... ..........
OXA-149         .......... .......... .......... .......... .......... ..........
OXA-78          .......... .......... .......... .........A .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   125        135        145        155        165        175
OXA-98          GDAMKASAIP VYQDLARRIG LELMSNEVKR VGYGNADIGT QVDNFWLVGP LKITPQQEAQ
OXA-77          .......... .....P.... .......... .......... .......... ..........
OXA-68          .......... .......... .......... .......... .......... ..........
OXA-91          .......... .......... .......... .......... .......... ..........
OXA-117         .......... .......... .......... .......... .......... ..........
OXA-150         .......... .......... .........H .......... .......... ..........
OXA-128         .......... .......... .......... .......... .......... ..........
OXA-144         .......... .......... .......... .......... .......... ..........
OXA-116         .......... .......... .......... .......... .......... ..........
OXA-149         .......... .......... .........H .......... .......... ..........
OXA-78          .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   185        195        205        215        225        235
OXA-98          FAYKLANKTL PFSQKVQDEV QSMLFIEEKN GNKIYAKSGW GWDVNPQVGW LTGWVVQPQG
OXA-77          .......... .......... .......... .......... .......... ..........
OXA-68          .......... ....E..... .......... .......... .......... ..........
OXA-91          .......... .......... .......... .......... ....D..... ..........
OXA-117         .......... .......... .......... .......... .......... ..E.......
OXA-150         .......... .......... .......... .......... .......... ..........
OXA-128         .......... ....E..... .......... .......... .......... ..........
OXA-144         .......... ....E..... ..I....... .......... .......... ..........
OXA-116         .......... .......... .......... .......... ....D..... ..........
OXA-149         .......... ...P...... .......... .......... .......... ..........
OXA-78          .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|
                   245        255        265        275
OXA-98          NIVAFSLNLE MKKGIPSSVR KEITYKSLEQ LGIL*
OXA-77          .......... .......... .......... ....*
OXA-68          .......... .......... .......... ....*
OXA-91          .......... .......... .......... ....*
OXA-117         .......... .......... .......... ....
OXA-150         .......... .......... .......... ....*
OXA-128         .......... .......... .......... ....*
OXA-144         .......... .......... .......... ....*
OXA-116         .......... .......... .......... ....
OXA-149         .......... .......... .......... ....*
OXA-78          .......... ....T..... .......... ....*
```

Appendix B.

Published papers.

Eleven novel OXA-51-like enzymes from clinical isolates of *Acinetobacter baumannii*

10.1111/j.1469-0691.2007.01828.x

Class D OXA-51-like carbapenemases are chromosomally encoded and appear to be intrinsic to *Acinetobacter baumannii* [1]. These enzymes are weak carbapenemases, and it has been suggested that they only confer carbapenem resistance if an additional promoter is provided by the insertion of IS*Aba1* upstream of the structural gene [2]. Following recent reports in *CMI* of new variants of the OXA-51-like subgroup of class D carbapenemases [3,4], we wish to report the identification of 11 more novel bla_{OXA-51}-like variants in clinical isolates of *A. baumannii*.

A collection of 60 clinical *A. baumannii* isolates of diverse worldwide origin was screened for bla_{OXA-51}-like genes using primers OXA-69A and OXA-69B, which amplify a 975-bp product containing the entire bla_{OXA-51}-like coding sequence [5]. Amplification products were sequenced on a 3730 DNA Analyzer (Applied Biosystems, Warrington, UK) and were compared using BLAST and Multalin software. Isolates producing a band of *c.* 2000 bp, as the result of an insertion upstream of the bla_{OXA-51}-like gene, were re-analysed using primers preABprom+ [6] and OXA-69B to produce a 1189-bp product, which was then sequenced as above.

Sequence comparison revealed 11 novel bla_{OXA-51}-like sequences, encoding 11 novel enzymes, designated OXA-79, OXA-80, OXA-82, OXA-104 and OXA-106–OXA-112 (http://www.lahey.org/studies/webt.asp). The origins of the isolates encoding these enzymes are listed in Table 1. These novel enzymes differ from all currently known enzymes by between one and four amino-acids (Table 1), sharing 99% amino-acid identity. The bla_{OXA-79}, bla_{OXA-80}, $bla_{OXA-106}$, $bla_{OXA-107}$, $bla_{OXA-109}$, $bla_{OXA-110}$ and $bla_{OXA-112}$ genes each differ from the gene encoding the closest related enzyme by a single nucleotide, resulting in a single amino-acid change. The nucleotide sequence of bla_{OXA-82} differs from bla_{OXA-66} by two nucleotides, one of which is silent ($T_{435} \rightarrow A$), and $bla_{OXA-111}$ has three silent substitutions compared to bla_{OXA-51} ($C_{90} \rightarrow T$, $T_{216} \rightarrow A$ and $T_{507} \rightarrow C$). The sequences of $bla_{OXA-104}$ and $bla_{OXA-108}$ differ from that of bla_{OXA-95} by 11 and five nucleotides, respectively, eight ($C_{54} \rightarrow T$, $C_{369} \rightarrow T$, $A_{390} \rightarrow G$, $G_{426} \rightarrow A$, $C_{549} \rightarrow T$, $C_{610} \rightarrow T$, $G_{720} \rightarrow A$ and $G_{732} \rightarrow A$) and one ($C_{328} \rightarrow T$) of which are silent. All amino-acid changes were located outside the class D carbapenemase motifs [7]. All isolates producing the larger *c.* 2000-bp product with primers OXA-69A and OXA-69B also yielded a 1189-bp product with the second set of primers, indicating that these isolates had an IS*Aba1* insertion upstream of the bla_{OXA-51}-like gene. Sequencing confirmed that the bla_{OXA-79}, bla_{OXA-80}, bla_{OXA-82} (in both isolates), $bla_{OXA-107}$ (in all isolates), $bla_{OXA-108}$, $bla_{OXA-109}$ and $bla_{OXA-110}$ (in both isolates) genes each had an IS*Aba1* insertion 7 bp upstream of the structural gene. A detailed analysis of the genotypic relationships among these isolates and the influence of the IS*Aba1* insertions on the expression of carbapenem resistance will be published separately.

Since the discovery of OXA-51 in 2004, the number of related enzymes in this group has now

Table 1. Amino-acid changes resulting in 11 novel enzymes

Related enzyme	Amino-acid change	Novel enzyme	Origin of isolates
OXA-66	$Trp_{222} \rightarrow Gly$ ($T_{664} \rightarrow G$)	OXA-79	UK
OXA-66	$Pro_{130} \rightarrow Leu$ ($C_{389} \rightarrow T$)	OXA-80	UK
OXA-66	$Leu_{167} \rightarrow Val$ ($C_{499} \rightarrow G$)	OXA-82	Turkey, USA
OXA-95	$Phe_{7} \rightarrow Leu$ ($C_{21} \rightarrow A$) $Asp_{117} \rightarrow Asn$ ($G_{349} \rightarrow A$) $Lys_{146} \rightarrow Asn$ ($G_{438} \rightarrow T$)	OXA-104[a]	USA
OXA-70	$His_{198} \rightarrow Asp$ ($C_{592} \rightarrow G$)	OXA-106	Estonia
OXA-69	$Leu_{167} \rightarrow Val$ ($C_{499} \rightarrow G$)	OXA-107	Poland, Slovenia
OXA-95	$Phe_{7} \rightarrow Leu$ ($C_{21} \rightarrow A$) $Ala_{25} \rightarrow Thr$ ($G_{73} \rightarrow A$) $Ala_{156} \rightarrow Thr$ ($G_{466} \rightarrow A$) $Leu_{167} \rightarrow Val$ ($C_{499} \rightarrow G$)	OXA-108[a]	Poland
OXA-66	$Pro_{130} \rightarrow Gln$ ($C_{389} \rightarrow A$)	OXA-109	UK
OXA-69	$Ile_{129} \rightarrow Leu$ ($A_{385} \rightarrow C$)	OXA-110	Poland
OXA-51	$Thr_{24} \rightarrow Ser$ ($A_{70} \rightarrow T$) $Val_{48} \rightarrow Ala$ ($T_{143} \rightarrow C$) $Lys_{209} \rightarrow Met$ ($A_{626} \rightarrow T$)	OXA-111	Belgium
OXA-69	$Ser_{246} \rightarrow Pro$ ($T_{736} \rightarrow C$)	OXA-112	UK

[a]OXA-104 and OXA-108 are equally distant from OXA-65 and OXA-95, with Asn225 → Asp in place of Phe7 → Leu. Nucleotide changes are listed in parentheses.

increased to at least 39, and they appear to be ubiquitous in *A. baumannii* [3,5,7,8]. While the insertion of IS*Aba1* upstream of bla_{OXA-51}-like genes has been associated with resistance to carbapenems, not all isolates contain such an insertion [2]. OXA-51 and OXA-69 have been shown to hydrolyse carbapenems poorly [5,7], and it would be of interest to determine whether the sequence diversity of this group is a result of selection for greater activity against the carbapenems.

NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

The novel sequences reported in this study are deposited in the GenBank nucleotide database under accession numbers EU019534 (OXA-79), EU019535 (OXA-80), EU019536 (OXA-82), EF581285 (OXA-104), EF650032 (OXA-106), EF650033 (OXA-107), EF650034 (OXA-108), EF650035 (OXA-109), EF650036 (OXA-110), EF650037 (OXA-111) and EF650038 (OXA-112).

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Mumps vaccine failure or vaccination scheme failure?

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We read with interest the study by Atrasheuskaya *et al.* [1] concerning vaccine failure in cases of mumps during 2002–2004 in Novosibirsk, Russia. According to Atrasheuskaya *et al.* [1], the question of whether these cases were caused by an absence of sufficient titres of antibody in response to vaccination, or were caused by the absence of neutralising antibody specific for the causative mumps virus strain, cannot be answered conclusively. On the basis of lessons learnt from a descriptive epidemiological investigation of a mumps outbreak in 214 young adults in Austria during 2006, we hypothesise that susceptibility to mumps infection is less likely to result from a vaccine failure than from a vaccination scheme failure, i.e., administration of only one of the two recommended doses of vaccine.

Active immunisation with the Jeryl Lynn strain of attenuated mumps virus vaccine has been available in Austria since 1974, when a bivalent mumps–measles (MM II) vaccine produced by Merck Sharp & Dohme was introduced as part of the national childhood immunisation programme. In 1994, the bivalent vaccine was replaced by a trivalent mumps, measles and rubella (MMR) vaccine produced by Pasteur Merieux Connaught (containing the Jeryl Lynn strain). Since 2001, the Priorix MMR vaccine (Glaxo Smith Kline) has been in use, which contains the RIT 4385 mumps strain derived from the Jeryl Lynn strain. The vaccination regimen includes two doses, with the first dose

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OXA-51-like β-lactamases and their association with particular epidemic lineages of *Acinetobacter baumannii*

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ABSTRACT

Sixty diverse clinical *Acinetobacter baumannii* isolates of worldwide origin were assigned to sequence groups, based on a multiplex PCR for the *ompA*, *csuE* and $bla_{OXA\text{-}51\text{-}like}$ genes. The majority (77%) of isolates belonged to sequence groups 1 and 2 (SG1 and SG2), with sequence group 3 (SG3) and non-grouped isolates accounting for the remainder. The isolates were not closely related according to pulsed-field gel electrophoresis (PFGE), and the majority were sensitive to imipenem and meropenem. The construction of a linkage map of OXA-51-like β-lactamase sequence relationships revealed two closely related clusters of enzymes, one focused around OXA-66 and the other around OXA-69. Isolates belonging to SG1 encoded an enzyme from the OXA-66 cluster, while those belonging to SG2 encoded an enzyme from the OXA-69 cluster. All SG3 isolates encoded OXA-71, which does not form part of a close enzyme grouping. Major multinational lineages accounted for a significant proportion of *A. baumannii* clinical isolates, and the evolution of the OXA-51-like enzymes appears to be an ongoing process.

Keywords *Acinetobacter baumannii*, $bla_{OXA\text{-}51\text{-}like}$, carbapenemase, lineages, linkage map, sequence groups

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INTRODUCTION

Acinetobacter baumannii is an important Gram-negative nosocomial pathogen responsible for serious infections in immunocompromised patients, particularly in intensive care units [1,2]. Infections are often difficult to treat because of the development of antimicrobial resistance, and particularly because of the emergence of carbapenem-hydrolysing β-lactamases, since carbapenems are now the drugs of choice for the treatment of *Acinetobacter* infections [3,4]. Carbapenem-resistant *A. baumannii* strains have been described as endemic since 1997 [5], and carbapenem resistance in *A. baumannii* has been described as a global sentinel event for emerging antimicrobial resistance [4]. In particular, the identification of three major lineages (termed European clones I, II and III), prevalent in hospitals across Europe, highlights the ability of successful lineages of this organism to disseminate widely [6–8].

Since the first report of an OXA-type carbapenemase in an *A. baumannii* isolate from 1985 [9], reports of class D β-lactamases have become common, and their contribution to high-level carbapenem resistance within strains of *A. baumannii* has been demonstrated [9,10]. The class D β-lactamase OXA-51 was first identified in *A. baumannii* in 2004 [11], and minor variations in the sequence encoding OXA-51 have subsequently been reported, constituting the OXA-51-like subgroup of enzymes [12–15]. It has been suggested that $bla_{OXA\text{-}51\text{-}like}$ genes are ubiquitous in *A. baumannii*, and that insertion of IS*Aba1* upstream of the genes may provide a promoter to enhance gene expression, potentially contributing to increased levels of resistance to carbapenems [14,16]. As these genes are apparently ubiquitous and unique to *A. baumannii*, it has been proposed that identification of this species can be based simply on the detection of an OXA-51-like enzyme [17].

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Sequence typing of 31 *A. baumannii* isolates, predominantly from the UK, using specific regions of $bla_{\text{OXA-51-like}}$ genes, the *ompA* gene (encoding the porin outer-membrane protein A) and the *csuE* gene (encoding a product important in a pilus chaperone–usher secretion system), has revealed three distinct lineages or sequence groups [18]. Interestingly, each sequence group was found to correspond to three predominant European lineages designated as European clones I, II and III. Also, three $bla_{\text{OXA-51-like}}$ alleles, corresponding to the three main sequence groups, were identified as $bla_{\text{OXA-66}}$, $bla_{\text{OXA-69}}$ and $bla_{\text{OXA-71}}$, respectively.

There are currently 37 members of the OXA-51-like group of enzymes, varying in structure by between one and 16 amino-acids. The present study aimed to investigate the relationships among the OXA-51-like enzyme family, together with the association of these enzymes with particular clonal groupings found among epidemic lineages and temporally diverse isolates of *A. baumannii* obtained from worldwide sources.

MATERIALS AND METHODS

Bacterial isolates

Sixty *A. baumannii* isolates were collected between 1982 and 2006 from hospitals worldwide (Fig. 1). Isolates were initially identified in the individual hospital laboratories using standard microbiological techniques, and were then confirmed as members of the *A. baumannii* complex [1] by tRNA fingerprinting [19].

PCR amplification and sequence analysis of $bla_{\text{OXA-51-like}}$ genes

All primers used in this study are listed in Table 1. DNA extraction was performed by boiling up to three colonies in 50 μL of sterile distilled water for 10 min. PCRs were performed in 50-μL volumes containing 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, nuclease-free bovine serum albumin 0.1 mg/mL, Triton X-100 0.1% v/v, 1.5 mM $MgCl_2$, 800 μM PCR nucleotide mix, and 1.25 U of *Pfu* DNA polymerase (Promega, Southampton, UK). The primers OXA-69A and OXA-69B [12] were used to amplify a 975-bp fragment containing the $bla_{\text{OXA-51-like}}$ gene under the following conditions: 95°C for 2 min, followed by 30 cycles of 95°C for 1 min, 48°C for 40 s and 72°C for 3 min, followed by 72°C for 6 min. For isolates that produced a product larger than 975 bp because of the presence of IS*Aba1* upstream of the $bla_{\text{OXA-51-like}}$ gene, the primer preABprom+ [20] was used with OXA-69B to produce a 1189-bp product under the same cycling conditions, except that the annealing temperature was increased to 53°C. Reactions were carried out in a Px2 Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) using primer concentrations of 25 pmol/μL and 0.5 μL of template DNA. PCR products were analysed on agarose 1.5% w/v gels stained with ethidium bromide, and were then scanned using the Diversity Database software image-capturing system (Bio-Rad, Hemel Hempstead, UK). Products were purified using a QIAquick PCR Purification Kit (Qiagen, Crawley, UK) and were sequenced in both directions on a 3730 DNA Analyzer (Applied Biosystems, Warrington, UK). Sequences were analysed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and MultAlin (http://bioinfo.genopole-toulouse.prd.fr/multalin/) software.

Pulsed-field gel electrophoresis (PFGE)

All isolates were typed by PFGE [21] following digestion of intact genomic DNA with *Apa*I (Promega). DNA fragments were separated on agarose 1% w/v gels in 0.5× TBE buffer (1× TBE buffer comprises 89 mM Tris, 89 mM boric acid, 2 mM EDTA) at 14°C using a CHEF DRII apparatus (Bio-Rad) with 6 V/cm, pulsed from 5 to 35 s, for 24 h. Gels were stained with ethidium bromide and scanned using the Diversity Database software image-capturing system. Analysis of the gels was performed using BioNumerics v.4.0 (Applied Maths, Sint-Martins-Latem, Belgium). Similarity was calculated using the Dice coefficient with a tolerance of 1.3% and the unweighted pair-group method using arithmetic averages (UPGMA).

MICs

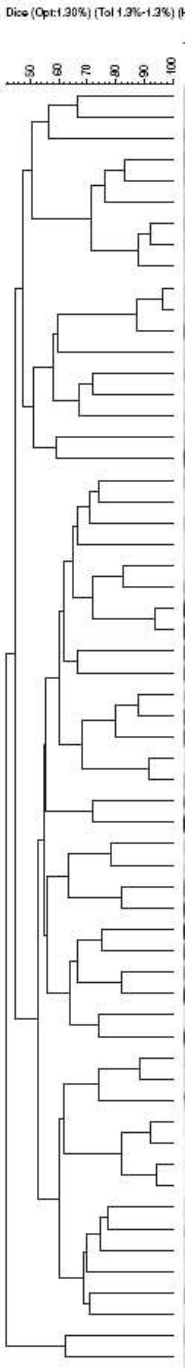
All isolates were tested for their susceptibility to imipenem and meropenem. MICs were determined by doubling dilutions in agar, according to the British Society for Antimicrobial Chemotherapy (BSAC) methodology [22]. The results were interpreted according to the guidelines of the BSAC [23]. *Pseudomonas aeruginosa* NCTC 10662, *Escherichia coli* NCTC 10418 and *Staphylococcus aureus* NCTC 6571 were used as quality control strains.

Sequence groups

Multiplex PCRs for identification of the *ompA*, *csuE* and $bla_{\text{OXA-51-like}}$ sequence groupings were performed as described by Turton *et al.* [18], except that each reaction used a Ready-to-Go PCR Bead (GE Healthcare Life Sciences, Little Chalfont, UK) containing pre-formulated PCR buffer, dNTPs and *Taq* polymerase in a final reaction volume of 25 μL.

OXA-51-like enzyme linkage map

All publicly available OXA-51-like amino-acid sequences were obtained from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). Sequences were analysed using MultAlin software (http://bioinfo.genopole-toulouse.prd.fr/multalin/) and all amino-acid variations were recorded. OXA-69 was chosen as a starting point in constructing the map, as this was the enzyme found in the oldest isolate in this study (strain A1, isolated in 1982). The map was subsequently re-drawn with OXA-65 as a starting point (see Results). Branches were constructed for each enzyme by listing the amino-acid changes from OXA-65, from the most common across all enzymes first, to the least common last. The branches were drawn in order, from the enzymes with the fewest differences from OXA-65 first, through to the enzymes with the highest number of differences. Branches with the same



Isolate	Origin	IMI	MER	Enzyme	IS*Aba1*	STG
A1	Nottingham, United Kingdom	0.25	0.5	OXA-69	–	2
A384	Oslo, Norway	4	2	OXA-69	–	2
A388	Ioannina, Greece	1	2	OXA-92	–	New
A230	Nottingham, United Kingdom	0.25	1	OXA-66	–	1
A387	Ioannina, Greece	4	4	OXA-66	–	1
A25	Nottingham, United Kingdom	0.25	0.5	OXA-66	–	1
A4	Nottingham, United Kingdom	0.5	0.5	OXA-66	–	1
A6	Nottingham, United Kingdom	0.25	0.5	OXA-66	–	1
A24	Nottingham, United Kingdom	0.12	0.25	OXA-66	–	1
A13	Rotterdam, The Netherlands	1	2	OXA-71	–	3
A20	Paris, France	0.25	1	OXA-71	–	3
A478	LUH 5875 European clone III	0.25	2	OXA-71	–	3
A37	Singapore	0.25	0.25	OXA-64	–	New
A397	Thessaloniki, Greece	4	4	OXA-66	–	1
A47	Berlin, Germany	0.5	1	OXA-66	–	1
A297	RUH 875 European clone I	0.25	0.5	OXA-69	–	2
A392	Freiburg, Germany	8	8	OXA-66	–	1
A60	Buenos Aires, Argentina	0.25	1	OXA-65	–	1
A187	Johannesburg, South Africa	0.12	1	OXA-68	–	New
A216	Sheffield, United Kingdom	0.5	1	OXA-66	–	1
A332	Bilbao, Spain	64	128	OXA-66	–	1
A94	Barcelona, Spain	8	4	OXA-71	–	3
A140	Cologne, Germany	0.25	0.5	OXA-66	–	1
A320	RUH 134 European clone II	0.12	0.5	OXA-66	–	1
A369	Oviedo, Spain	>128	>128	OXA-66	–	1
A401	Taiwan	8	16	OXA-82	+	1
A329	Barcelona, Spain	32	128	OXA-71	–	3
A418	Wroclaw, Poland	4	8	OXA-66	–	1
A186	Johannesburg, South Africa	0.25	0.5	OXA-66	–	1
A343	Chesterfield, United Kingdom	1	2	OXA-109	+	1
A335	Lincoln, United Kingdom	0.12	0.5	OXA-69	–	2
A14	Venlo, The Netherlands	0.25	2	OXA-69	–	2
A167	Bosnia	0.25	0.5	OXA-69	–	2
A473	Warsaw, Poland	4	4	OXA-108	+	1
A474	Warsaw, Poland	4	2	OXA-66	–	1
A365	London, United Kingdom	0.5	0.5	OXA-66	–	1
A371	Prague, Czech Republic	8	32	OXA-83	+	1
A368	Mansfield, United Kingdom	0.25	0.5	OXA-112	–	2
A457	Estonia	1	2	OXA-106	–	New
A390	Pleven, Bulgaria	8	16	OXA-69	–	2
A52	Freiburg, Germany	4	8	OXA-69	–	2
A424	Croatia	4	16	OXA-107	+	2
A472	Warsaw, Poland	4	8	OXA-107	+	2
A15	Dordrecht, The Netherlands	0.5	0.5	OXA-69	–	2
A92	Barcelona, Spain	0.25	1	OXA-69	–	New
A404	Wroclaw, Poland	2	4	OXA-110	+	2
A407	Wroclaw, Poland	0.5	4	OXA-110	+	2
A443	Ljubljana, Slovenia	2	8	OXA-107	+	2
A480	Nottingham, United Kingdom	0.25	0.25	OXA-66	–	1
A484	Nottingham, United Kingdom	8	32	OXA-83	+	1
A481	Nottingham, United Kingdom	0.5	2	OXA-66	–	1
A482	Nottingham, United Kingdom	0.5	1	OXA-66	–	1
A135	Ghent, Belgium	0.12	0.25	OXA-111	–	New
A442	Sofia, Bulgaria	1	2	OXA-69	–	2
A479	Pakistan	8	8	OXA-69	–	2
A376	Feldkirch, Austria	0.25	1	OXA-68	–	New
A380	London, United Kingdom	16	32	OXA-66	–	1
A95	Trieste, Italy	0.06	0.12	OXA-98	–	New
A377	Regensburg, Germany	4	2	OXA-71	–	3
A63	Buenos Aires, Argentina	0.12	0.5	OXA-69	–	2

Fig. 1. Pulsed-field gel electrophoresis profiles for strains of *Acinetobacter baumannii*, showing imipenem and meropenem MICs, OXA-51-like β-lactamase types, the presence of IS*Aba1* upstream of the $bla_{\text{OXA-51-like}}$ gene, and sequence group types. IMI, imipenem; MER, meropenem; STG, sequence type group.

Table 1. Primers used in this study

Primer	Sequence (5′–3′)	Reference
OXA-69A	CTAATAATTGATCTACTCAAG	[12]
OXA-69B	CCAGTGGATGGATGGATAGATTATC	[12]
preABprom+	GACCTGCAAGAAGCGCTGC	[20]
Group1ompAF306	GATGGCGTAAATCGTGGTA	[18]
Group1and2ompAR660	CAACTTTAGCGATTTCTGG	[18]
Group1csuEF	CTTTAGCAAACATGACCTACC	[18]
Group1csuER	TACACCCGGGTTAATCGT	[18]
Gp1OXA66F89	GCGCTTCAAAATCTGATGTA	[18]
Gp1OXA66R647	GCGTATATTTTGTTTCCATTC	[18]
Group2ompAF378	GACCTTTCTTATCACAACGA	[18]
Group2csuEF	GGCGAACATGACCTATTT	[18]
Group2csuER	CTTCATGGCTCGTTGGTT	[18]
Gp2OXA69F169	CATCAAGGTCAAACTCAA	[18]
Gp2OXA69R330	TAGCCTTTTTTCCCCATC	[18]

changes within them were merged to produce the fewest number of branches possible. Amino-acid changes were not reversed within the same branch.

RESULTS

Sequence groups and $bla_{\text{OXA-51-like}}$ sequence analysis

All experimental results are summarised in Fig. 1. SG1 formed the largest group, accounting for 47% of the isolates. The second largest group was SG2, containing 30% of the isolates, and SG3 was the smallest group, representing 10% of the isolates. Representatives of the three European lineages were found in separate sequence groups, with EC1 belonging to SG2, EC2 belonging to SG1, and EC3 belonging to SG3, as described previously [18]. Eight (13%) isolates did not belong to any of the three major sequence groups, and produced novel combinations of products in the two multiplex PCRs.

The majority of isolates yielded a $bla_{\text{OXA-66}}$, $bla_{\text{OXA-69}}$ or a $bla_{\text{OXA-71}}$ sequence; $bla_{\text{OXA-66}}$ was found in 22 (37%) isolates, $bla_{\text{OXA-69}}$ was found in 14 (23%) isolates, and $bla_{\text{OXA-71}}$ was found in six (10%) isolates. Sequences corresponding to the other enzymes were found in one or two isolates only, except for $bla_{\text{OXA-107}}$, which was found in three isolates. The sequence from isolate A92 had five nucleotide substitutions compared with the published $bla_{\text{OXA-69}}$ sequence ($G_{426} \rightarrow A$, $C_{474} \rightarrow A$, $C_{511} \rightarrow T$, $G_{540} \rightarrow A$ and $T_{801} \rightarrow C$), but none of these resulted in an amino-acid change. The representative isolates of EC1 (A297), EC2 (A320) and EC3 (A478) encoded OXA-69, OXA-66 and OXA-71, respectively. Ten isolates (17%) were found to contain an IS*Aba1* element seven nucleotides upstream of their $bla_{\text{OXA-51-like}}$ gene.

PFGE profile analysis revealed that the isolates varied considerably, with very few distinct groups. Six pairs of isolates and two groups of three isolates clustered at ≥87% similarity, which is a cut-off value that has been suggested for use in identifying isolates belonging to the same epidemic strain [24]. The majority (52%) of isolates shared ≤78% similarity. Some isolates with different $bla_{\text{OXA-51-like}}$ sequences, and belonging to different sequence groups, were more similar to one another, according to PFGE analysis, than they were to isolates that contained the same $bla_{\text{OXA-51-like}}$ gene and belonged to the same sequence group. Thus, isolate A186, which belonged to SG1 and contained a $bla_{\text{OXA-66}}$ gene, shared 80% identity with isolate A335, which belonged to SG2 and contained a $bla_{\text{OXA-69}}$ gene. Similarly, isolate A368, which belonged to SG2 and contained a $bla_{\text{OXA-112}}$ gene, shared 82% identity with isolate A457, which contained a $bla_{\text{OXA-106}}$ gene and belonged to a novel sequence group. When isolates were from the same location, contained the same $bla_{\text{OXA-51-like}}$ gene, and belonged to the same sequence group, they tended to form more closely related clusters. This can be seen with isolates A480, A484, A481 and A482 from Nottingham, UK. These isolates represent a dominant clone in the particular hospital from which the isolates were taken.

Susceptibility testing

The majority (60%) of isolates included in the study were susceptible to both imipenem and meropenem according to BSAC criteria. Resistance to imipenem or meropenem or both was seen in nine isolates. Of the four imipenem- and meropenem-resistant isolates, three were from Spain, and all contained one of the three most common enzymes; A332, A380 and A369 had a $bla_{\text{OXA-66}}$ sequence, while A329 had a $bla_{\text{OXA-71}}$ sequence. Of the five isolates that were resistant only to meropenem, three encoded less common enzymes that were closely related to OXA-66 or OXA-69: A371 encoded OXA-83, A401 encoded OXA-82, and A424 encoded OXA-107.

OXA-51-like enzyme linkage map

The linkage map revealed that the enzymes formed distinct groupings (Fig. 2). Three very closely inter-related enzyme groups, with each

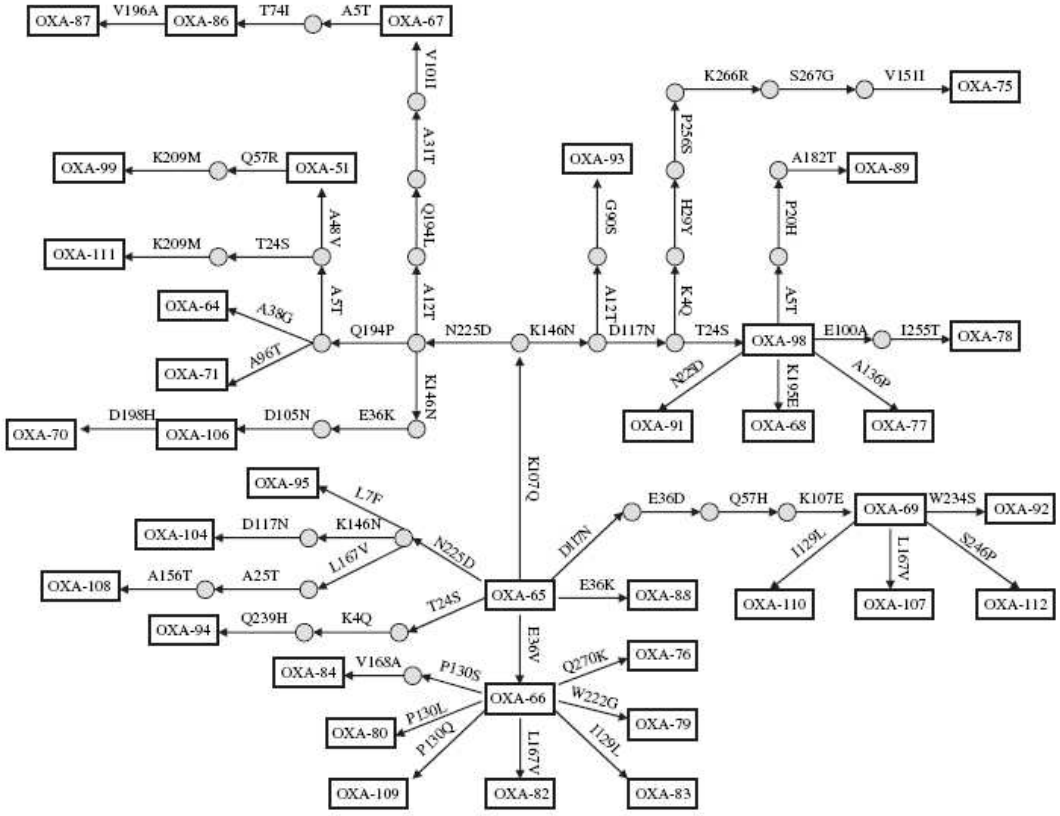


Fig. 2. OXA-51-like β-lactamase enzyme linkage map. Amino-acid substitutions are labelled with respect to OXA-65.

member separated by one or two amino-acid differences, were formed around OXA-66, OXA-69 and OXA-98. Two other major enzyme groups were not as closely inter-related. In the group containing OXA-71, differences at five positions separated OXA-71 and OXA-99. The second group had OXA-108 separated from OXA-104, also by changes at five positions. OXA-67, OXA-86 and OXA-87 formed a chain rather than a cluster. The three enzymes representative of SG1, SG2 and SG3 were not closely related, with changes at four positions between OXA-66 and OXA-69, five positions between OXA-66 and OXA-71, and seven positions between OXA-69 and OXA-71. The OXA-66 cluster was the largest group, with ten members (OXA-66, OXA-65, OXA-88, OXA-76, OXA-109, OXA-82, OXA-83, OXA-84, OXA-79 and OXA-80). A member of this group, OXA-65, appeared to form a central hub within the map from which all the major branches radiated. The OXA-69 cluster had five members (OXA-69, OXA-92, OXA-107, OXA-110 and OXA-112), as did the OXA-98 cluster (OXA-98, OXA-91, OXA-68, OXA-77 and OXA-78). In contrast, OXA-71 was closely related only to OXA-64 and, unlike OXA-66, OXA-69 and OXA-98, was not a group hub, but was instead found on a branch tip.

DISCUSSION

Construction of a map showing the relationships among the OXA-51-like enzymes revealed that the representative enzymes found in SG1, SG2 and SG3 belonged to separate groups. OXA-66 and OXA-69 appear to be ancestral to their groups of very closely related enzymes. Such a high degree of conservation of the OXA-66 cluster and OXA-69 cluster, relative to the rest of the OXA-51-like enzymes, is consistent with these enzymes

belonging to highly successful lineages such as EC1 and EC2. By multiplex PCR sequence typing, the isolates encoding the members of the OXA-66 cluster were all assigned to SG1, and all except two (A92 and A388) of the isolates encoding the OXA-69 cluster were assigned to SG2, demonstrating that they contain the same *ompA* and *csuE* alleles as well as $bla_{\text{OXA-51-like}}$ alleles. This suggests that the similarities within these isolates extend not just to the $bla_{\text{OXA-51-like}}$ β-lactamase gene, but also to other genes potentially involved in the successful colonisation and infection of patients.

Two isolates, A92 and A388, encoded OXA-69-clustered enzymes, but did not belong to SG2. Both isolates were positive within the group 2 multiplex PCR for the $bla_{\text{OXA-51-like}}$ amplicon, but both differed from other SG2 isolates in that they failed to yield a *csuE* amplicon in the group 2 multiplex PCR (data not shown). These two isolates, and the six other isolates which were not assigned to a sequence group, may represent strains that are capable of causing outbreaks of infection in particular locations, but that are unable to establish themselves more widely in competition with other more successful epidemic lineages. Comparisons of such isolates with members of the more prevalent lineages may provide insights into the factors involved in successful epidemic spread. It would be interesting to determine the sequence groups of a range of isolates representing all of the OXA-51-like enzymes in order to determine whether there are other small sequence groups, such as SG3, which may represent future highly successful lineages such as SG1 and SG2.

The enzyme linkage map shows that OXA-65 is a central hub from which all of the major groupings radiate. To progress from OXA-69 to the other major groupings would require the unlikely event of multiple substitutions at the same amino-acid position. This suggests that $bla_{\text{OXA-65}}$, or an as yet undiscovered closely related gene sequence, may be ancestral to all $bla_{\text{OXA-51-like}}$ genes in *A. baumannii*. Previously, isolates from 1982, 1983 and 1984 were found to encode OXA-51, OXA-78 and OXA-89, respectively [14]. These enzymes are not closely related to OXA-66 or OXA-69, which suggests that much of the diversity of the OXA-51-like enzymes had evolved before *A. baumannii* was identified as a significant nosocomial pathogen.

The enzyme linkage map demonstrates that the OXA-66 cluster forms the largest group of closely related enzymes. In the present study, isolates encoding enzymes in the OXA-66 cluster formed the largest group, accounting for 45% of all isolates. The second largest enzyme group, the OXA-69 cluster, formed the second largest isolate group, accounting for 33% of isolates. Isolates encoding OXA-71 accounted for only 10% of the total. In this study of mainly European *A. baumannii* isolates, SG1 was by far the most prolific, suggesting that EC2 is part of an extensive lineage that is well-established and exhibits little variation in certain genes involved in virulence. This is mirrored in the enzyme linkage map, with the OXA-66 cluster being the largest, but also being highly conserved. The same is true for SG2, although this lineage, containing EC1, is slightly less prolific. SG3/EC3 is different, in that it is associated with only one enzyme, OXA-71. SG3 was not nearly as prevalent in this study as the other two sequence groups, although this clone may well have been more highly represented had more isolates been included from the Iberian peninsular. While the enzyme linkage map shows a closely related group surrounding OXA-98, only three isolates in this study encoded enzymes belonging to this cluster, indicating that these enzymes are not associated with predominant lineages. The most recently identified enzymes largely branch from the main cluster foci, suggesting that the evolution of these enzymes is progressing in real-time.

IS*Aba1* was detected upstream of $bla_{\text{OXA-51-like}}$ genes in only ten isolates. Nine of these were resistant or had intermediate MICs of at least one carbapenem; however, these accounted for only 37.5% of such isolates, demonstrating that this feature was not responsible for conferring carbapenem resistance in the majority of isolates. The IS*Aba1* sequences were all found upstream of more recently identified, branch-tip enzymes. The reason for this is unknown. The ability of mobile elements such as IS*Aba1* to insert upstream of the $bla_{\text{OXA-51-like}}$ genes presents the possibility of their past or future mobilisation.

As might be expected from such a broad range of isolates, there was a large degree of genomic variation among the isolates. It is interesting to note that isolates can be more closely related, according to PFGE, to an isolate of a different sequence group than they are to other members of

their own sequence group. Previously it was shown that isolates of major outbreak strains with related PFGE patterns all corresponded to a specific sequence group, while the sporadic isolates did not [18]. The data presented here indicate that non-major outbreak isolates can also be assigned to one of the three identified sequence groups, although this is not always the case, and that isolates of the same sequence group are not always related according to PFGE. *A. baumannii* is known to contain a variety of mobile elements, such as IS*Aba1*, often in multiple copies, and the movement of such elements, along with the possibility of natural transformation, as seen in other *Acinetobacter* spp., could contribute to the genomic variation observed [25,26].

If $bla_{\text{OXA-51-like}}$ genes are immobile, it would be expected that isolates containing closely related enzymes would generally be more closely related to one another than to isolates containing distantly related enzymes. In addition, sequence typing of two further genes such as *ompA* and *csuE* should reduce still further any differences seen between PFGE typing and sequence typing. The apparent disparity between the two typing schemes in these data raises important questions as to the appropriateness of these schemes for *A. baumannii*. Further work to determine their suitability is required.

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OXA-type β-lactamases in *Acinetobacter baumannii*: emerging from the shadow of the extended-spectrum β-lactamases

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The extended-spectrum β-lactamases have evolved owing to the extensive use of the oxyimino-cephalosporins, and have greatly reduced the treatment options for serious infections. *Acinetobacter baumannii* is becoming an increasingly important multi-resistant nosocomial pathogen due to the carriage of class D OXA-type β-lactamases. The OXA-23-like, OXA-40-like and OXA-58-like β-lactamases confer carbapenem resistance and are increasingly being found in association with a diversity of mobile genetic elements. High prevalence of OXA-40-like enzymes on the Iberian Peninsula, OXA-58-like enzymes across Europe and very high prevalence of OXA-23-like enzymes in South America and Asia are of concern. The intrinsic OXA-51-like enzymes of *A. baumannii* may confer carbapenem resistance when overexpressed, and form a large enzyme family. Similar patterns are beginning to be seen for the OXA-type β-lactamases, which were previously seen in the emergence of the extended-spectrum β-lactamases, the continuation of which would pose a grave threat to the antibiotic era.

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Introduction

In the 1980s, widespread use was made of a new class of antibiotics, the oxyimino-cephalosporins. These drugs had been developed in response to the spread of plasmid-encoded β-lactamases TEM-1, TEM-2 and SHV-1 that conferred resistance to earlier cephalosporins and penicillins [1]. However, the huge selective pressure exerted by the use of the oxyimino-cephalosporins led to the rapid emergence of variants of the TEM and SHV enzymes that had expanded their substrate spectrum to include these new drugs. These early enzymes have now been joined by another major group, the CTX-M-type β-lactamases, along with a host of smaller groups such as the VEB, PER, GES and some OXA enzymes to form a collection of enzymes referred to as the extended-spectrum β-lactamases (ESBLs) [2]. The continuing evolution of the ESBLs to confer resistance to the majority of the β-lactams has left fewer options for the treatment of serious infections, with the carbapenems becoming one of the drugs of choice due to their broad spectrum of activity, including ESBL producers [3].

Acinetobacter baumannii is a nosocomially acquired Gram-negative bacterium that is emerging as an important pathogen due to the increasing rates of multidrug resistance [4]. The bacterium is most problematic in ICU wards where it can cause ventilator-associated pneumonia, bacteraemia and urinary tract infections. Some studies have reported attributable mortality of *A. baumannii* infection of up to 43% [5]. One of the few remaining antibiotic classes that are effective in treating *A. baumannii* infections is the carbapenems. However, increasing rates of resistance to the carbapenems are being described, largely due to the presence of four groups of molecular class D OXA-type β-lactamases: the OXA-23-like, OXA-40-like, OXA-51-like and OXA-58-like enzymes [6]. Some of the structural, evolutionary and

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epidemiological features of these enzymes show worrying similarities to patterns observed during the emergence of the ESBLs.

The OXA-23-like enzymes

The enzyme OXA-23, originally named ARI-1, was first identified in an *A. baumannii* strain from Scotland, isolated in 1985, with a minimum inhibitory concentration (MIC) for imipenem of 16 $mg l^{-1}$ [7]. The enzyme was shown to confer resistance to imipenem, and subsequent sequence analysis identified the enzyme as belonging to the Ambler class D β-lactamases [8]. Two further enzymes with 99% amino acid identity to OXA-23 have been identified and named OXA-27 and OXA-49. OXA-27 was identified in an isolate from Singapore, isolated between 1995 and 1997, and OXA-49 was associated with an isolate from China and the sequence was published online in 2003 (accession number AY288523) [9]. Very recently, three further enzymes named OXA-102, OXA-103 and OXA-105 have been identified in isolates of *Acinetobacter radioresistens* [10]. These six enzymes constitute the OXA-23-like enzyme group. Limited kinetic analyses of the OXA-23, OXA-27 and OXA-49 enzymes demonstrate that they hydrolyse aminopenicillins and oxacillin, and weakly hydrolyse oxyimino-cephalosporins and carbapenems [7,9]. However, differences were observed between OXA-23 and OXA-27, with OXA-23 having a higher activity against cephaloridine and oxacillin and less activity against carbapenems than OXA-27 [9].

The OXA-23-like enzymes have been found in a variety of *Acinetobacter* species, as well as in isolates of *Proteus mirabilis*, and can be chromosomally encoded or located on a variety of different plasmids (Table 1) [11,12]. The G+C content of the $bla_{OXA-23-like}$ genes is 37.6–37.9%, which is outside the 39–47% range for the *A. baumannii* genome, suggesting that these genes have evolved in another species [4]. The recent identification of bla_{OXA-23}, $bla_{OXA-102}$, $bla_{OXA-103}$ and $bla_{OXA-105}$ genes on the chromosome of several *A. radioresistens* isolates suggests that the OXA-23-like enzymes may have originated in that species [10]. Isolates encoding OXA-23 have been found worldwide and are frequently associated with outbreaks of hospital infection [13–17]. Of particular concern is the recent increase in reports of carbapenem-resistant *A. baumannii* isolates encoding OXA-23 from South America and south-east Asia. A survey of 542 *A. baumannii* isolates from 10 institutions in Colombia found an average of 33.6% of isolates resistant to a carbapenem, and in a subset of 66 of these resistant isolates, 65 were positive by PCR for bla_{OXA-23} [13]. In a teaching hospital in China, rates of carbapenem resistance have increased from 5% in the period 1993–2003 to greater than 50% in isolates from ICU wards and 20% in

Table 1. Features of the OXA-type β-lactamases of *A. baumannii*.

Enzyme group	Group members	Genetic location[a]	Host species	References
OXA-23-like	OXA-23, OXA-27, OXA-49, OXA-102, OXA-103, OXA-105	C and P (40, 70, 150 kb)	*A. baumannii*, *A. junii*, *A. radioresistens*, *Acinetobacter* genomic species 3, *Proteus mirabilis*, *Acinetobacter* phenon 5, *Acinetobacter* phenon 6/ct 13TU, *Acinetobacter* genomic species 13TU, *Acinetobacter* genomic species 10/11	[10,12,13,16–18]
OXA-40-like	OXA-40, OXA-25, OXA-26, OXA-72	C and P	*A. baumannii*, *A. haemolyticus*, *Acinetobacter* genomic species 3	[9,18,23,26,28]
OXA-51-like	OXA-51, OXA-64 to OXA-71, OXA-75 to OXA-80, OXA-82 to OXA-84, OXA-86 to OXA-95, OXA-98 to OXA-100, OXA-104, OXA-106 to OXA-113, OXA-115 to OXA-117, OXA-120 to OXA-127	C	*A. baumannii*	[19,30,32–39] (http://www.lahey.org/studies/)
OXA-58-like	OXA-58, OXA-96, OXA-97	C and P (10, 15, 20, 21, 22, 25, 27, 30, 40, 50, 51, 100, 150 kb)	*A. baumannii*, *A. junii*, *Acinetobacter* genomic species 3, *Acinetobacter* genospecies 13TU, *Acinetobacter* phenon 6/ct 13TU	[11,15,16,18,43,45,47]

[a]C, chromosomal location; P, plasmid location.

non-ICU isolates in 2004; and a subsequent study of 221 resistant isolates from 11 institutions, including 117 isolates from the same teaching hospital, found 97.7% to be positive for a $bla_{\text{OXA-23-like}}$ gene [18].

In vivo, the OXA-23 β-lactamase contributes towards resistance to amoxicillin, ticarcillin, meropenem and imipenem. When transformed into susceptible $bla_{\text{OXA-23}}$-negative recipient strains, the enzyme confers intermediate to low level resistance to carbapenems, with the higher of these values achieved in combination with overexpression of the RND-type AdeABC efflux pump. However, when a naturally occurring plasmid containing the $bla_{\text{OXA-23}}$ gene is electrotransformed into the same recipient strains, moderate to high MICs are achieved of 16 to more than 32 $mg\,l^{-1}$, with the highest values again found in the recipient overexpressing the AdeABC efflux pump, indicating the involvement of other genetic factors associated with the $bla_{\text{OXA-23}}$ gene in conferring resistance [16]. It has been suggested that the insertion sequence IS*Aba1* may act as a promoter to increase $bla_{\text{OXA-23}}$ expression in a similar manner to the role it plays in increasing bla_{ampC} expression, as the insertion sequence has been found upstream of $bla_{\text{OXA-23}}$ in more than 97% of carbapenem-resistant isolates [14,19,20]. Recent studies have shown that IS*Aba1* inserted 25 bp upstream of the $bla_{\text{OXA-23}}$ gene contains sequences which can act as promoters for its expression [21,22]. Another insertion sequence, IS*Aba4*, has similarly been found 25 bp upstream of $bla_{\text{OXA-23}}$ and which contains promoter sequences [21]. As well as potentially increasing expression, these insertion sequences may explain the widespread prevalence of $bla_{\text{OXA-23}}$ genes both in *A. baumannii* and in other *Acinetobacter* species as they form putative transposons named Tn*2006* (containing IS*Aba1*) and Tn*2007* (containing IS*Aba4*) [21].

The OXA-40-like enzymes

OXA-40, originally named OXA-24, was first identified in *A. baumannii* isolates responsible for an outbreak of infection in a hospital in Spain in 1997 [23]. Two further enzymes named OXA-25 and OXA-26, both with more than 99% amino acid identity to OXA-40, were identified in isolates from Belgium and Spain isolated between 1995 and 1997 respectively [9]. A fourth enzyme also with more than 99% amino acid identity to OXA-40, named OXA-72, was identified in an isolate from Thailand and the sequence published online in 2004 (accession number AY739646). These four enzymes constitute the OXA-40-like enzyme group.

Kinetic studies carried out on OXA-40, OXA-25 and OXA-26 demonstrate that these enzymes are capable of hydrolysing penicillins, have weak activity against carbapenems and very weak activity against some cephalosporins [9,23,24]. All three enzymes show greater hydrolytic activity against imipenem than meropenem, with OXA-40 being the most active. The recent description of the crystal structure of OXA-40 provides insights into the substrate specificity of the enzyme [25]. Analysis of the arrangement of the active site elements demonstrates similarities between OXA-40 and the structures of other non *A. baumannii* class D oxacillinases, and therefore does not explain the different substrate specificity of increased carbapenem and reduced oxacillin hydrolysis of this enzyme. However, two residues, Tyr-112 and Met-223, interact to form a hydrophobic tunnel that restricts access to the active site of the enzyme. Modelling predicts that molecules such as oxacillin, which has a large methylphenylisoxazolyl moiety, will therefore have their access to the active site reduced, whereas imipenem and meropenem with their small hydroxyethyl moieties are able to gain access more easily. Experiments on the OXA-40 β-lactamase with the Tyr-112 and Met-223 residues replaced with alanine demonstrated that the more enclosed hydrophobic environment surrounding the active site is also responsible for correctly orientating carbapenems for hydrolysis, which may help explain why the enzyme can hydrolyse carbapenems, whereas class D oxacillinases cannot.

Despite the weak activity of the purified enzyme against the carbapenems, isolates encoding an OXA-40-like enzyme typically return MICs of more than 16 $mg\,l^{-1}$ [26]. A study by Heritier *et al.* [16] demonstrated that, *in vivo*, the OXA-40 enzyme confers resistance to carbapenems as well as raises MICs to penicillins and cephalosporins. However, when transformed into susceptible reference *A. baumannii* strains, the enzyme only confers intermediate to low level resistance, with the higher of these values associated with an isolate overexpressing the AdeABC efflux pump, suggesting that a combination of mechanisms is required to achieve the high levels of resistance seen in clinical isolates.

The G+C content of the $bla_{\text{OXA-40-like}}$ genes is 33.9–34.3%, which differs from the content of the *A. baumannii* genome of 39–47%, suggesting that these genes originated in another species [4]. The $bla_{\text{OXA-40-like}}$ genes have been found in three species of *Acinetobacter* and to be both chromosomally and plasmid encoded (Table 1). Although it has been found on a variety of different plasmids, the specific plasmid sizes have not been reported. Isolates encoding the enzymes have been reported in China [18], Belgium [9] and the USA [27], but the major geographic foci of the enzymes are Spain and Portugal [26,28,29]. It is of concern that, on the Iberian Peninsula, there appears to be a high prevalence of the $bla_{\text{OXA-40-like}}$ genes. A recent study of 83 carbapenem-resistant *A. baumannii* isolates from 12 hospitals in Spain found 42% of isolates to be positive for a $bla_{\text{OXA-40-like}}$ gene [26]. A second recent study [29] from a hospital in Portugal found that, of 222 imipenem-resistant

A. baumannii collected over the period of January 2001 to October 2004, 36.6% carried a bla_{OXA-40} gene, the majority of which were associated with plasmid DNA, which was also found in *Acinetobacter haemolyticus* isolates in the same institution. The identification of a high percentage of $bla_{OXA-40-like}$ genes on plasmids presents the worrying possibility of these genes becoming more globally established.

The OXA-51-like enzymes

The OXA-51-like β-lactamases are an enzyme group that are intrinsic and ubiquitous in *A. baumannii*, and have not been found in any other species [30,31]. The first enzyme of the group, OXA-51, was reported in 2004 in isolates obtained from Argentina in 1996 [32], and a large number of related enzymes have since been discovered. The OXA-51-like enzymes now represent one of the largest groups of β-lactamases, with 51 enzymes currently identified [19,30,32–39]; http://www.lahey.org/studies (accessed 15 January 2008). The $bla_{OXA-51-like}$ genes have generally been found to be chromosomally encoded and nontransferable. However, in one report, PCRs for $bla_{OXA-51-like}$ genes using plasmid DNA as a template were positive, and the $bla_{OXA-51-like}$ gene could be cured from these isolates [40], and a second report mentions that the investigators were able to transfer a $bla_{OXA-51-like}$ gene to *Escherichia coli* [34], suggesting that it may be possible for these genes to be plasmid-borne. The G+C content of the genes is 38.4–39.9%, which is similar to the content of the *A. baumannii* genome of 39–47%, lending further weight to the suggestion that these genes are native to *A. baumannii* [4].

The contribution of these enzymes to resistance in *A. baumannii* has yet to be fully resolved. Kinetic analysis has only been performed on two enzymes: OXA-51 and OXA-69 [32,37]. Both enzymes demonstrate poor hydrolysis of oxacillin and cloxacillin, a feature that has been observed in carbapenem-hydrolysing oxacillinases [9,23]. High K_m values for imipenem and meropenem in OXA-69 indicate a poor affinity for these substrates, but OXA-51 has a low K_m for imipenem, suggesting a much higher affinity. However, OXA-69 weakly hydrolyses imipenem and meropenem, whereas OXA-51 weakly hydrolyses imipenem but not meropenem. The contribution of the enzymes to carbapenem resistance has been difficult to determine. Every member of the species encodes a $bla_{OXA-51-like}$ gene, yet only a subset demonstrates carbapenem resistance. However, comparisons of imipenem-resistant and imipenem-susceptible isolates from Taiwan demonstrated that the resistant isolates (MICs for imipenem and meropenem of 8 and 32 $mg l^{-1}$ respectively to >32 $mg l^{-1}$ for both antibiotics) had increased expression levels of their $bla_{OXA-51-like}$ genes relative to the susceptible isolates (MICs for imipenem and meropenem of 1–3 $mg l^{-1}$ and 0.383–1.5 $mg l^{-1}$ respectively), and that all of the resistant isolates were positive by PCR for an IS*Aba1* sequence 7bp upstream of the $bla_{OXA-51-like}$ gene, whereas the sensitive isolates were not [41]. Similar results were obtained in a study of Spanish isolates, in which two isolates of the same pulsetype differed in $bla_{OXA-51-like}$ gene expression with the isolate containing IS*Aba1* 7bp upstream of the gene having a higher level of expression than the isolate with IS*Aba1* elsewhere in the genome [26]. The insertion of IS*Aba1* upstream of $bla_{OXA-51-like}$ genes in the opposite orientation provides a promoter region for the $bla_{OXA-51-like}$ genes, and it has been proposed that this leads to upregulation of gene expression resulting in increased levels of resistance [19]. However, it is unclear whether upregulation of the $bla_{OXA-51-like}$ gene alone is sufficient or responsible for clinically significant levels of resistance to carbapenems or whether it requires other resistance mechanisms working in concert such as reduced outer membrane permeability [41].

There is a large degree of variety within the OXA-51-like enzymes, with members differing by up to 16 amino acids (Fig. 1). Within the OXA-51-like enzymes, there are subgroups or clusters of enzymes that are associated with certain epidemic lineages. The large group of enzymes clustered around the OXA-66 β-lactamase are found in isolates belonging to an *A. baumannii* lineage including the prevalent European clone 2, whereas those clustered around the OXA-69 enzyme are found in another lineage encompassing European clone 1. The OXA-71 enzyme is associated with European clone 3 [42]. The most commonly identified enzymes are those of the OXA-66 cluster, which are particularly highly represented in South America and Asia [18,30,38,42]. Enzymes of the OXA-69 cluster are also common, particularly in eastern Europe [42]. Additionally, OXA-71 is regularly identified and due to its association with European clone 3, is often identified in isolates from the Iberian Peninsula [42,43]. It is unknown whether sequence variations in the OXA-51-like enzymes contribute to increased resistance or an altered spectrum resulting in certain enzymes being selected, thus altering allele frequencies, or if certain enzymes are more prevalent due to their association with a particularly widespread lineage that is successful due to other factors.

The OXA-58-like enzymes

The enzyme OXA-58 was first identified in an *A. baumannii* clinical isolate in 2003 in France [44]. The enzyme shares 48% and 49% amino acid identity with OXA-23 and OXA-40 respectively, and was localised to a 30 kb plasmid. Two enzymes closely related to OXA-58 have since been identified. An *A. baumannii* isolated in 1996 from a hospital in Singapore contained a β-lactamase named OXA-96, which differs from the OXA-58 enzyme

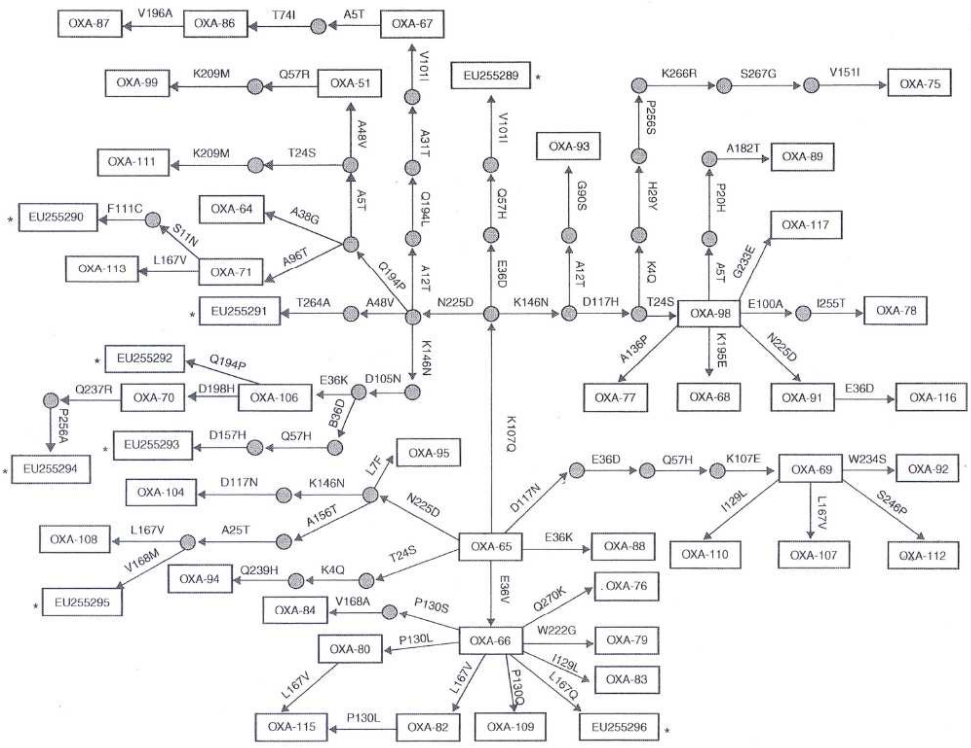

Fig. 1. OXA-51-like enzyme linkage map. Amino acid substitutions are labelled with respect to OXA-65. Modified from Evans *et al.* [42]. Accession numbers for enzyme amino acid sequences that were not named at the time of going to press are marked with asterisks. OXA-90 and OXA-100 are not included as their sequences are not currently available.

by a methionine substituting isoleucine at position 161 [38], and an enzyme named OXA-97 which differs from OXA-58 by a glycine substituting an alanine at position 53 has been identified in *A. baumannii* isolates in a hospital in Tunisia (accession number EF102240). These three enzymes constitute the OXA-58-like enzyme group.

Kinetic analysis of OXA-58 has shown that it has similar properties to the other OXA-type carbapenemases of *A. baumannii*. The purified enzyme has weak activity against penicillins and imipenem, very weak activity against meropenem, and some activity against cephalothin and cefpirome, but not against ceftazidime or cefotaxime [44]. However, OXA-58 hydrolyses imipenem twice as efficiently as OXA-40. When a plasmid carrying the bla_{OXA-58} gene was transformed into two susceptible *A. baumannii* reference strains, it resulted in greatly increased MICs for amoxicillin and ticarcillin but only very small increases in carbapenem MICs, to which the strains remained susceptible. However, when a plasmid containing the bla_{OXA-58} gene from a clinical isolate was transformed into the same reference strains, the MICs for carbapenems increased significantly in the strain overexpressing the AdeABC efflux pump (MICs for imipenem and meropenem of 32 mg l^{-1}). In the second strain, MICs were higher than when the strain was transformed with artificial OXA-58 plasmid, although the strain remained sensitive (MICs of 2 mg l^{-1}) [16]. This demonstrates that isolates carrying a $bla_{OXA-58-like}$ gene may require a combination of factors to achieve high-level carbapenem resistance.

OXA-58-like enzymes have been found in a range of *Acinetobacter* species and are usually plasmid borne, although chromosomal location has been described (Table 1) [11,45]. The G+C content of the $bla_{OXA-58-like}$ genes is 37.4–37.5%, which is slightly lower than the *A. baumannii* genome content of 39–47%, indicating that these genes may have originated in another species [4]. Isolates carrying $bla_{OXA-58-like}$ genes are most frequently reported from Europe, although the genes have been reported in isolates from South America, Kuwait, China, Singapore and Australia [17,18,38,46,47]. Analysis of the genetic environment of bla_{OXA-58} has shown that the gene

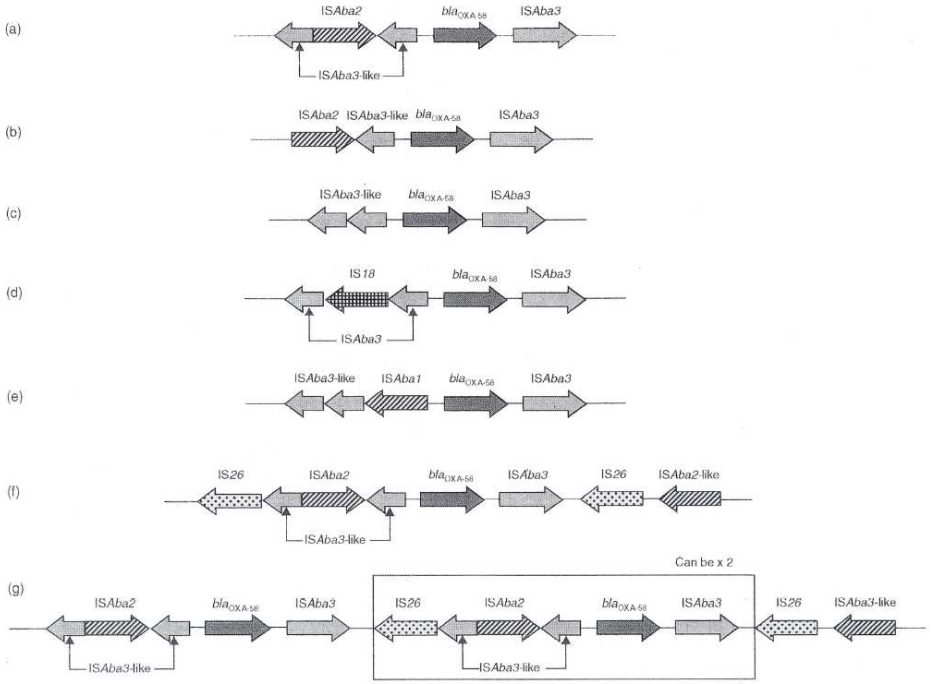

Fig. 2. Genetic elements surrounding *bla*$_{OXA-58}$. The IS*Aba3*-like element is missing the sequence for the last 20 C-terminal amino acids of the TnpA transposase as well as the right inverted repeat from IS*Aba3*. The structure in (d) with the complete IS*Aba3* element interrupted by an IS*18* sequence may represent a composite transposon formed by the IS*Aba3* elements upstream and downstream of the *bla*$_{OXA-58}$ gene. The IS*Aba2*-like element is as IS*Aba2* except that a 1 bp insertion within its InsB transposase sequence has introduced a stop codon. The boxed region in (g) has been found to be directly repeated in one isolate, possibly by imperfect duplicative transposition of the putative transposon formed by the IS*26* elements. Genes and transcription orientations are shown by horizontal arrows. Diagram is not to scale. Data for (a–e) have been taken from Poirel and Nordmann [48], and data for (f) and (g) have been taken from Bertini *et al.* [49].

is associated with a variety of mobile elements, which are summarised in Fig. 2 [48,49]. The presence of IS*Aba1* upstream of *bla*$_{OXA-58}$ provides a promoter identical to that provided when IS*Aba1* inserts upstream of the *bla*$_{ampC}$ gene (Fig. 2e). Hybrid promoters are present between IS*Aba2* and the IS*Aba3*-like element immediately upstream of *bla*$_{OXA-58}$ (Fig. 2a, b, f, g), and also between IS*18* and the IS*Aba3* element that it interrupts (Fig. 2d). The IS*Aba3*-like elements alone also provide a promoter (Fig. 2c). The presence of these promoters upstream of *bla*$_{OXA-58}$ in clinical *A. baumannii* isolates may result in increased expression levels leading to high levels of carbapenem resistance. It has been suggested that the unusual IS*Aba3*-like structure surrounding *bla*$_{OXA-58}$ indicates that the gene was acquired through a recombination event [48]. Insertion sequences also appear to be involved in altering the copy number of the *bla*$_{OXA-58}$ gene, as duplication of the gene mediated by IS*26* elements has been observed (Fig. 2f, g). The study examined three clonal isolates, one containing one copy of *bla*$_{OXA-58}$ (as shown in Fig. 2f), one containing two copies (as shown in Fig. 2g), and one containing three copies (as shown in Fig. 2g, except that the boxed region was duplicated). The MICs for imipenem for the three isolates were 16, 32 and 128 mg l^{-1} respectively [49]. This demonstrates that as well as increased expression due to promoters provided by insertion sequences, duplication of the *bla*$_{OXA-58}$ gene mediated by IS*26* elements result in an increase in resistance to carbapenems.

The ESBL connection

The ESBLs are generally defined as β-lactamases with a broad spectrum able to hydrolyse oxyimino-cephalosporins at a rate of at least 10% of that for benzylpenicillin and are inhibited by clavulanate, although not all enzymes that are generally considered as ESBLs meet all of these

criteria [1]. The first ESBL to be identified was SHV-2 in 1982, a mutant of SHV-1 that had an expanded spectrum and was able to hydrolyse oxyimino-cephalosporins. This was soon followed by the identification of TEM-3, an expanded-spectrum variant of TEM-1 [1]. More recently, there has been a rapid emergence of a third group of ESBLs, the CTX-M enzymes, and these three groups of enzymes contain by far the largest number of variants within the ESBLs, with 161 TEM, 105 SHV and 69 CTX-M variants currently identified [50]; http://www.lahey.org/studies (accessed 15 January 2008).

Many features of the ESBLs' mechanisms, evolution and epidemiology bear a close resemblance to features of the OXA-type β-lactamases of *A. baumannii*. The first and the most obvious similarity between the two are the structures of the enzyme groups. The OXA-type β-lactamases of *A. baumannii* form groups of related enzymes that differ structurally from the other members of their group by a few amino acids. The OXA-23-like, OXA-40-like and OXA-58-like groups only contain a few members, similar to the PER-type, VEB- type and GES-type ESBLs, whereas the OXA-51-like enzymes form a much larger group more similar to the TEM- type, SHV- type and CTX-M-type ESBLs [2,51]. However, although the effects on substrate hydrolysis of the structural changes in the majority of the ESBLs are known, this is not the case for the OXA-type β-lactamases. Although some differences in enzyme kinetics have been observed within the members of the OXA-23-like group, the OXA-40-like group and the OXA-51-like group, none of these differences have been assigned to specific amino acid changes. The data are particularly poor for the OXA-51-like enzymes, in which, out of a large group of 51 enzymes identified so far, only OXA-51 and OXA-69 have been examined in any detail. There are also problems with determining the evolutionary history of the OXA-type β-lactamases as no parental enzymes have been identified. As the G+C content of the $bla_{\text{OXA-23-like}}$, the $bla_{\text{OXA-40-like}}$ and the $bla_{\text{OXA-58-like}}$ genes are less than that of the *A. baumannii* genome as a whole, it is thought that these genes have been acquired from other species. Recent data suggest that this may be *A. radioresistens* in the case of the $bla_{\text{OXA-23-like}}$ genes. However, the $bla_{\text{OXA-51-like}}$ genes are thought to have originated in *A. baumannii*. These enzymes may have evolved from a common ancestor, in a similar manner to the evolution of the SHV-type ESBLs from a chromosomal enzyme in *Klebsiella pneumoniae* [52], or from several parental enzymes, possibly explaining the specific subgrouping that is seen within this enzyme group, in a similar although less distinctive manner to the evolution of the CTX-M-type ESBLs from enzymes from different *Kluyvera* species [53]. The more recently identified OXA-51-like enzymes tend to be located on the extremities of the enzyme linkage map (Fig. 1), suggesting that these enzymes are evolving in real time, but whether this evolution is mirroring that of the TEM, SHV and CTX-M ESBLs, with amino acid changes resulting in altered rates or spectrums of hydrolysis is currently unknown.

Kinetic studies of the OXA-type β-lactamases of *A. baumannii* have shown the enzymes to have weak activity against carbapenems, but isolates expressing these enzymes are resistant. Although other resistance mechanisms encoded by these isolates appear to contribute to resistance, it may be that kinetic studies are underestimating enzyme hydrolysis *in vivo*. A study of the ESBL OXA-14 observed biphasic kinetics, but with a burst size that was dependent on enzyme concentration. It was demonstrated that higher hydrolysis rates at higher enzyme concentrations were probably due to the stabilization of the enzyme by dimer formation at high concentrations, and that *in vivo* the concentration is high enough that the enzyme will exist as a dimer [54]. Enzyme kinetic studies are usually carried out at concentrations at which the enzyme would exist as a monomer. Biphasic kinetics have been recorded for OXA-23-like and OXA-58-like enzymes, and there is evidence that OXA-51-like enzymes may exist as dimers [9,37,44]. It is, therefore, possible that the catalytic rate and efficiency of the OXA-type β-lactamases is much higher *in vivo* than that reported by current in-vitro studies.

As described earlier, the OXA-type β-lactamases of *A. baumannii* are increasingly being found associated with insertion sequences. Some insertion sequences provide promoters that may enhance gene expression; some form putative transposons and may play a role in gene mobility and, in one example involving OXA-58, appear to have been responsible for the duplication of the $bla_{\text{OXA-58}}$ gene. Similar features have been observed for the ESBLs. The insertion sequence IS*Ecp1* found upstream of many of the $bla_{\text{CTX-M}}$ genes provides typical -35 and -10 promoter regions in its 3′ end and, similarly, promoters provided by IS*Pa12* upstream of $bla_{\text{PER-1}}$ enhance expression of the β-lactamase, resembling the promoters provided by IS*Aba4* upstream of $bla_{\text{OXA-23-like}}$ genes, IS*Aba1* upstream of $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-51-like}}$ and $bla_{\text{OXA-58-like}}$ genes, and IS*Aba2*, IS*Aba3* and IS*18* upstream of $bla_{\text{OXA-58-like}}$ genes [50,55,56]. Duplication of a resistance gene, or the presence of several different alleles of the gene in one isolate is a feature that has been observed for bla_{SHV} genes, and higher copy number has been related to increased levels of resistance, as has been recorded with IS*26*-mediated duplication of $bla_{\text{OXA-58}}$ [57,58]. Insertion sequences involved in ESBL mobilization are frequently seen. In *Pseudomonas aeruginosa* and *A. baumannii* isolates, the $bla_{\text{PER-1}}$ gene has been found as part of a composite transposon Tn*1213*, formed by IS*Pa12* and IS*Pa13* upstream and downstream of the gene respectively [56]. The same transposon was found in *Providencia stuartii*, although with the additional insertion of an IS*Prst1* sequence between the $bla_{\text{PER-1}}$ gene and IS*Pa13*. The $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-58}}$ genes have already been identified on putative composite transposons and

the potential of these genes to spread to other species in a manner frequently seen with ESBLs is concerning. Of particular concern is the recent identification of bla_{OXA-58} associated with IS*26* sequences. IS*26* is commonly associated with ESBLs. It has been proposed that IS*26* was responsible for the mobilization of bla_{SHV} alleles from the *K. pneumoniae* genome on at least two occasions during their evolution. IS*26* forms composite transposons containing bla_{SHV} genes and containing bla_{VEB-1} and bla_{OXA-10} genes (Tn*2000*), has been found upstream of IS*Ecp1*-bla_{CTX-M} elements, and was recently identified upstream of a bla_{SHV-5} gene in *A. baumannii* [39,52,59,60]. IS*26* has also been implicated in the formation of multiresistant loci on plasmids from *Salmonella enterica* serovar Typhimurium, *K. pneumoniae* and *E. coli* through acquisition of resistance genes by homologous recombination [61]. This presents the worrying scenario in which bla_{OXA-58} could be mobilised by IS*26* and spread to other species commonly expressing ESBLs such as *K. pneumoniae* or *E. coli*, wherein it may be able to confer carbapenem resistance.

Some epidemiological features of the ESBLs share similarities with the OXA-type β-lactamases. Many TEM-type ESBLs can be found right across Europe, whereas, in the USA, outbreaks are usually associated with TEM-10 or TEM-26. However, TEM-type enzymes are not very prevalent in southern South America or Asia, except in South Korea, where TEM-52 is commonly identified [62–65]. Conversely, of the SHV-type enzymes, SHV-5 and SHV-12 are the two that are most commonly encountered worldwide [62–65]. Perhaps most alarming is the recent explosion of isolates encoding enzymes of the CTX-M type. In Europe, enzymes belonging to the CTX-M-9 group are common in Spain, and are also found in Portugal, the UK and France, although they are far less common in the rest of Europe. CTX-M-15 is found across the continent, whereas CTX-M-3 is most often found in Eastern Europe. Within Europe, enzymes from the CTX-M-8 group and the CTX-M-25 group have only been reported in the UK [62]. In the USA, there have been no reports of outbreaks due to isolates encoding CTX-M-type enzymes [63]. However, in Argentina in 2002, CTX-M-2 was the most common ESBL in the country, whereas, in Asia, CTX-M-3 and CTX-M-14 are very common [64,65]. In the USA, the OXA-23-like, OXA-40-like and OXA-58-like enzymes have only begun to be reported relatively recently [66,67]. In Asia and South America, OXA-23-like enzymes are very common and are identified in isolates far more regularly than in Europe [13,17,18]. The OXA-58-like enzymes are most frequently identified in isolates from Europe, and OXA-40-like isolates are restricted mainly to the Iberian Peninsula [17,26]. Within the OXA-51-like enzymes, OXA-71 is also most frequently found on the Iberian Peninsula, whereas enzymes of the OXA-69 cluster are regularly found in Eastern Europe, and OXA-66 is very highly represented in south-east Asia [18,42]. The factors responsible for uneven geographic spread of the ESBLs and the OXA-type β-lactamases have not been clearly defined and they are likely many and varied with complex interactions, but it is reasonable to suggest that similar factors have led to the relatively low prevalence of β-lactamases in the USA, very high prevalence in South America and Asia, and complex distribution across Europe.

Conclusion

The ability of clinicians to treat *A. baumannii* infections is being impaired due to the increasing prevalence of carbapenem-resistant isolates, the majority of which is due to the carriage of OXA-type β-lactamases. The OXA-23-like β-lactamases confer high levels of carbapenem resistance *in vivo* and have been found in putative transposons. The bla_{OXA-23} gene has been identified in *P. mirabilis* as well as in a large number of *Acinetobacter* species, and the rapidly increasing prevalence of these genes, particularly in South America and Asia, is concerning. The OXA-40-like enzymes also confer resistance *in vivo*, and are particularly prevalent on the Iberian Peninsula. However, their location on several different plasmids may lead to increased prevalence elsewhere. Resistance due to the OXA-58-like enzymes is found globally, and the bla_{OXA-58} gene has been found to be associated with a variety of mobile elements. In addition to these, all *A. baumannii* possess a $bla_{OXA-51-like}$ gene that appears to confer carbapenem resistance when overexpressed. The OXA-51-like enzymes constitute one of the largest β-lactamase groups, although it is unknown whether the amino acid changes between members of the group lead to altered hydrolysis rates or substrate affinity. The factors leading to the emergence and success of the ESBLs such as widespread use of certain antibiotics, mutation to create new variants with altered substrate spectrum and association with mobile genetic elements may be responsible for the similar features now beginning to be seen with the OXA-type β-lactamases of *A. baumannii*. It is of great importance to determine whether such factors are acting upon the OXA-type β-lactamases because, if these enzymes emulate the ESBL model and spread across the Gram-negative bacteria, they could come to represent one of the largest threats to the antibiotic era yet seen.

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Acinetobacter baumannii: Emergence of Four Strains with Novel $bla_{\text{OXA-51-like}}$ Genes in Patients with Diabetes Mellitus

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Summary

Diabetic patients are 10 times more likely to develop *Acinetobacter baumannii* infections than the rest of the population. Carbapenems are considered one of the very few antibiotics left to treat infections caused by this organism. The aim of this work was to characterise *A. baumannii* strains isolated from diabetic patients and to investigate whether there is a relationship between certain strains and low-level-carbapenem resistance. Methods: Clinical samples were collected from diabetic patients in hospitals throughout Saudi Arabia from December 2006 to April 2007. API 20 NE, polymorphisms in the 16S-23S-rRNA intergenic region and the presence of a $bla_{\text{OXA-51-like}}$ gene were all used for identification. Susceptibility to antimicrobials was determined using agar dilution and disk diffusion methods. Pulsed-field gel electrophoresis (PFGE) coupled with sequence analysis of the $bla_{\text{OXA-51-like}}$ genes were used for strain characterization. Polymerase chain reaction (PCR) and multiplex PCR were used to screen for the presence and location of IS*Aba1* elements and $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-40-like}}$, and $bla_{\text{OXA-58-like}}$ genes respectively. Results: Twenty isolates were identified as *A. baumannii* and were all highly resistant to 38% of the antibiotics tested and the majority of isolates were also resistant to 50% of the remaining antibiotics. Four strains had low-level meropenem resistance (MIC 4 – 8 mg/L). All isolates were sensitive to imipenem and colistin. Nine strains possessed four novel $bla_{\text{OXA-51-like}}$ genes encoding β-lactamases designated OXA-90, OXA-130, OXA-131 and OXA-132, and four strains contained $bla_{\text{OXA-131}}$ with IS*Aba1* upstream of the gene structure. PFGE showed five separate clusters of OXA-51-like enzymes and the dissemination of strains carrying the four novel enzymes was clonal. This study showed that new strains of *A. baumannii* characterised by their new $bla_{\text{OXA-51-like}}$ gene have emerged. No genes encoding OXA-23-like, OXA-40-like, or OXA-58-like β-lactamases were found. Surveillance of *A. baumannii* harbouring the $bla_{\text{OXA-131-like}}$ gene may be an essential step in monitoring their carbapenem resistance phenotype and may assist in preventing their spread in diabetics.

Key words: ***Acinetobacter baumannii*, IS*Aba1* element, OXA-51-like β-lactamases, diabetes, diabetics, carbapenem, resistance.**

INTRODUCTION

Saudi Arabia is one of the top five countries with the highest prevalence of diabetes in the adult population according to the International Diabetes Federation in 2007 [1]. *Acinetobacter baumannii* is well recognized as an important pathogen responsible for serious infections associated with immunocompromised patients in intensive care and burn units (ICUs),[2-5] and infections caused by this organism are common in pa-

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tients with Diabetes Mellitus (DM) [6-9]. In addition, *A. baumannii* has the ability to acquire resistance to most antibiotics, including carbapenems, and pan-drug resistance is now emerging [10-12]. Many examples have been reported that carbapenem resistance has emerged in that local area [13-18]. Carbapenems, such as imipenem and meropenem, have a very broad spectrum of activity and have been the antibiotics of choice for treatment of infections caused by this pathogen [19]. Resistance to carbapenems is largely manifested by the class D OXA β-lactamases which comprise OXA-23-like, OXA-40-like, OXA-58-like and OXA-51-like [11] β-lactamases. OXA-51-like β-lactamases are present in all isolates of *A. baumannii* [20] and carbapenem resistance has been associated with this gene when the insertion sequence IS*Aba1* was located upstream of the gene structure, where it may provide a promoter sequence enhancing the expression of $bla_{OXA-51-like}$ genes [21]. As diabetic patients are vulnerable to *A. baumannii* infections [7], we hypothesised that they could be a fertile ground for species variation and/or antibiotic selection. Therefore, the aim of this work was to evaluate the epidemiology of *A. baumannii* in diabetics and to establish whether there is a link between particular strains and low-level carbapenem resistance.

MATERIALS AND METHODS

Clinical isolates

Clinical samples had been routinely collected from both hospital in-patients and community patients, all with diabetes mellitus, between December 2006 and April 2007 from different deep-sited infections. All were from four major hospitals and 20 medical centres in Saudi Arabia. Amongst these clinical samples, 20 isolates were identified as *A. baumanni*.

Phenotypic and genotypic identification

Initially the 20 isolates had been identified phenotypically by API 20 NE (BioMerieux, Marcy L'Etoile, France) as *A. calcoaceticus/A. baumannii* complex. Isolates were identified to the genomic level by restriction polymorphism in the 16S-23S rRNA intragenic region as described previously [22]. Concisely, DNA from each strain was obtained by emulsifying a single colony, obtained from an overnight growth on MacConkey agar (Oxoid, Basingstoke), in 25 µl sterilised distilled water in a micro centrifuge tube and then boiled for 10 minutes. The sequences of primer 1 (5′-TTG TAC ACA CCG CCC GTC A -′3) and primer 2 (5′- GGT ACT TAG ATG TTT CAG TTC -′3) were used to amplify a 975bp section of the gene [22]. The amplification reactions were performed in a volume of 50 µl and contained 10 mM Tris HCl (pH 8.3), 50 mM KCl, 200 mM deoxynucleotide, 0.5 mM of each primer, 3 mM $MgCl_2$, 2.5 U of *Taq* polymerase, and 10 to 100 ng of target DNA. PCR amplification mixtures were submitted to 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. Each amplification experiment included a blank, which contained all reagents with the exception of target DNA. Amplification products were analyzed by gel electrophoresis in 1.5% agarose gels.

Detection of β-lactamases by multiplex PCR

Multiplex PCR was used to screen for genes encoding OXA-51-like, OXA-23-like, OXA-40-like, and OXA-58-like enzymes in all 20 isolates. The primers pairs used were (5′-TAA TGC TTT GAT CGG CCT TG-′3) and (5′-TGG ATT GCA CTT CAT CTT GG-′3) to amplify a 353 bp fragment of the $bla_{OXA-51-like}$ gene, (5′-GAT CGG ATT GGA GAA CCA GA-′3) and (5′-ATT TCT GAC CGC ATT TCC AT-′3) to amplify a 501bp fragment of the $bla_{OXA-23-like}$ gene, (5′-GGT TAG TTG GCC CCC TTA AA-′3) and (5′-AGT TGA GCG AAA AGG GGA TT-′3) to amplify a 246bp fragment of the $bla_{OXA-40-like}$ gene, and (5′-AAG TAT TGG GGC TTG TGC TG-′3) and (5′-CCC CTC TGC GCT CTA CAT AC-′3) to amplify a 599bp fragment of the $bla_{OXA-58-like}$ gene. The amplification conditions for the multiplex PCR were the following: denaturation at 94°C for 5 min, and then 30 cycles at 94°C for 25 s, 52°C for 40 s and 72°C for 50s followed by a final extension at 72°C for 6 min as described previously by Woodford *et al* [19].

PCR amplifications were also performed with the primers OXA-69A (5′-CTA ATA ATT GAT CTA CTC AAG-′3) and OXA-69B (5′-CCA GTG GAT GGA TGG ATA GAT TAT C-′3) to obtain a 975bp product which contained the coding sequence of the entire $bla_{OXA-51-like}$ gene [23]. The amplification products were sequenced on an ABI3730 capillary sequencer (Applied Biosystems, Warrington, UK) and were analysed using BLAST and Multalin software. Isolates producing a band of more than c. 2000 bp resulting from an insertion upstream of the $bla_{OXA-51-like}$ gene were reanalysed by using the primers preABprom+ (5′-GAC CTG CAA AGA AGC GCT GC-′3) and OXA-69B to generate a 1189bp product [24], which was then sequenced.

Pulsed-field gel electrophoresis (PFGE)

All isolates were typed by PFGE analysis according a technique described by Miranda *et al* [25]. DNA obtained from bacteria was digested using Apa1 restriction endonuclease (Promega, Southampton, UK), and DNA fragments were separated on a 1% agarose gel in 0.5x TBE buffer using the CHEF DRII apparatus (Bio Rad, UK). The conditions used were the following: pulse time 5-35s at a field strength of 6V/cm for 24h at 14°C. The gel was stained by ethidium bromide and then the digital images were captured by Gel Doc 2000 (Bio-Rad, UK). All isolates were analysed using BioNumerics software version 4. Isolates that clustered together with a similarity of >85% were considered to belong to the same PFGE type.

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined for susceptibility to imipenem, meropenem, ertapenem, ciprofloxacin, gentamicin, oxacillin, piperacillin/tazobactam, ampicillin, amoxicillin-clavulanate, nalidixic acid, colistin, rifampicin, sulbactam, ofloxacin, chloramphenicol and tetracycline, using the agar dilution method based on the British Society for Antimicrobial Chemotherapy (BSAC) guidelines [26].

RESULTS

All isolates were highly resistant to ertapenem, oxacillin, ampicillin, sulbactam, chloramphenicol and tetracycline (*Table 1*). The majority of isolates were also resistant to ciprofloxacin, gentamicin, piperacillin/tazobactam, amoxicillin/clavulanate, nalidixic acid, rifampicin and ofloxacin. All isolates were susceptible to imipenem (0.25 – 2 mg/L) and colistin (0.25 - 0.5 mg/L) but four had low-level meropenem resistance (MIC 4 – 8 mg/L) (*Table 1*). Nine strains carried four novel $bla_{OXA-51-like}$ genes identified by the sequence comparison. The four novel β-lactamases were designated OXA-90, OXA-130, OXA-131 and OXA-132 (*Table 2*). Nine isolates were susceptible to imipenem, but four of them which had four novel enzymes were intermediate to meropenem. The strains encoding the four novel enzymes were from two hospitals and one medical centre, and were obtained from six males and three females aged between 46 to 93 years. One of these strains, which has the OXA-131 β-lactamase, possesses the insertion sequence IS*Aba1* inserted 7 base pairs upstream from the gene structure. The four novel enzymes differ from one to two amino-acids to the closest related enzymes, with 99% identity. The nucleotide sequence of $bla_{OXA-130}$ had an G→C change at nucleotide position 499, resulting in a Valine→Leucine substitution at amino-acid 167. In addition, $bla_{OXA-131}$ had two changes, A→G at nucleotide position 385, resulting in an Isoleucine→Valine substitution at amino-acid position 129, and C→A at nucleotide position 677, resulting in a Proline→Glutamine substitution at amino-acid position 226. The nucleotide sequence of $bla_{OXA-132}$ had a T→G change at position 789, resulting in an Isoleucine→Methionine substitution at amino-acid position 263. The sequence of bla_{OXA-90} differs from bla_{OXA-95} by six nucleotides, one of which is C→A at nucleotide position 21 resulting in a Phenylalanine→Leucine substitutions at amino-acid 7, and the other five nucleotide differences are silent (A_{15} → C, C_{328} → T, G_{501} → A, T_{636} → C and G_{720} → A) (*Table 2*). PFGE revealed five separate clusters of OXA-51-like β-lactamases. The novel enzyme OXA-131 was predominant, comprising one large group of four isolates from the main collection (isolates 3, 7, 8 and 15). OXA-89 was found only in five isolates from diabetic patients from the community, two of which grouped in one cluster (isolates 21 and 25). Just one isolate contained the OXA-130 β-lactamase. The OXA-132 and OXA-90 β-lactamase-containing strains each grouped in separate clusters with just two isolates in each (isolates 13 and 16, and 20 and 23 respectively), while two isolates encoding OXA-91 (isolates 27 and 29) were grouped together. Three isolates contained the widely-disseminated OXA-66 β-lactamase, two of which grouped in one cluster (isolates 19 and 22), and one isolate encoding OXA-78 was found (isolate 1) (*Figure 1*).

The sequence data reported in this study are deposited in the GenBank nucleotide database under accession numbers: EU547443 (OXA-90), EU547445 (OXA-130), EU547446 (OXA-131) and EU547447 (OXA-132).

TABLE 1 - *Antimicrobial susceptibility patterns of Acinetobacter baumannii isolates from diabetics (n=20).*

Antibiotics	No. of resistant isolates	MIC range (mg/L)	% resistance
Imipenem	0	0.25 – 2	0
Meropenem	4	4 – 8	20
Ertapenem	20	4 – 64	100
Ciprofloxacin	9	1 – 16	45
Gentamicin	4	32 – >128	20
Oxacillin	20	> 128	100
Pipericillin/tazobactam	11	32 – 128	55
Ampicillin	20	16 – > 128	100
Amoxicillin/clavulanate	18	16 – > 128	90
Nalidixic Acid	15	8 – > 128	75
Colistin	0	0.25 – 0.5	0
Rifampicin	16	4 – 32	80
Sulbactam	20	64 – > 128	100
Ofloxacin	14	1 – 32	70
Chloramphenicol	20	> 128	100
Tetracycline	20	2 – >128	100

DISCUSSION

This study identifies a new risk factor in diabetics. Emergence of new strains of *A. baumannii* isolated from patients with diabetes mellitus could reach epidemic levels [6]. The percentage of *A. baumannii* isolates from those patients having strains with novel OXA-51-like β-lactamases was high (45%). OXA-51-like β-lactamases may significantly contribute to carbapenem resistance [10,19,21,27], and this resistance correlates both with the survival of *A. baumannii* in diabetics and perhaps also in the development of dia-

TABLE 2 - *Nucleotide and amino-acid changes in four novel OXA-51-like enzymes.*

Isolate no.	Site	Source	Sex	Age	Related enzyme	Amino-acid changes	Nucleotide changes	Novel enzyme	IS*Aba1*
20 23	Sputum Urine	KFGH RMC	M F	65 46	OXA-95	Phe $_{7}$ → Leu	A $_{15}$ → C C $_{21}$ → A C $_{328}$ → T G $_{501}$ → A T $_{636}$ → C G $_{720}$ → A	OXA-90	–
2	Blood	KAHNG	M	93	OXA-108	Val $_{167}$ → Leu	G $_{499}$ → C	OXA-130	–
3	Blood	KAHNG	M	75					
7	Wound	KAHNG	F	76	OXA-66	Ile $_{129}$ → Val Pro $_{226}$ → Gln	A $_{385}$ → G C $_{677}$ → A	OXA-131	+
8	Ulcer	KAHNG	F	57					
15	Urine	KAHNG	M	87					
13	Urine	KAHNG	M	82	OXA-51	Ile $_{263}$ → Met	T $_{789}$ → G	OXA-132	–
16	Urine	KAHNG	M	70					

KAHNG: King Abdulaziz Hospital for National Guard - Alahssa
KFGH: King Fahad General Hospital - Hofuf
RMC: Rashdia Medical Centre - community of Almubarraz
(+): Presence of IS*Aba1* upstream of the $bla_{OXA-51-like}$ gene in these strains
(–): Lack of detection of IS*Aba1* upstream of the $bla_{OXA-51-like}$ gene in these strains

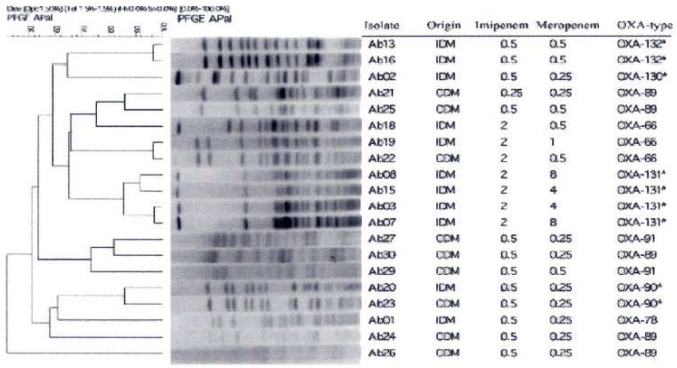

FIGURE 1 - Dendrogram showing results of dice coefficient based on PFGE of restricted DNA fragments of 20 isolates from diabetics. (*) Nine isolates assigned with four novel $bla_{OXA-51-like}$ genes. IDM: In-patient Diabetes Mellitus. CDM: Community Diabetes Mellitus.

betes [7]. On the other hand, a new study (unpublished paper) has revealed that there are certain gene variants that predispose people to develop diabetes, and these variations play a significant role in the development of a large spectrum of effects [28], which may include infections with *A. baumannii*. Nine representative isolates of *A. baumannii* from patients with diabetes mellitus were found to possess common gene variants encoding β-lactamases designated OXA-90, OXA-130, OXA-131 and OXA-132. A previous study described that all variations in amino-acids in OXA-51-like enzymes were outside of class D carbapenemase motifs [29], and this is consistent with the four novel enzymes identified in this study. The isolates with a PCR product larger than c. 2000bp using primers OXA-69A and OXA-69B produced a 1189bp product with the second set of primers [23]. The nucleotide sequences of these strains had the insertion sequence IS*Aba1* located 7 base pairs upstream from the open reading frame of the $bla_{OXA-131}$ gene (*Table 2*), which was the most frequent $bla_{OXA-51-like}$ gene (44%), compared to $bla_{OXA-130}$ (11%), $bla_{OXA-132}$ (22%) and bla_{OXA-90} (22%). Insertion sequence IS*Aba1* is widespread in *A. baumannii* with more than 13 copies

in one cell and, through mobilisation, can act as a 'moving switch' to upregulate expression of downstream genes [30]. The presence of IS*Aba1* upstream of $bla_{OXA\text{-}131\text{-}like}$ indicates that it may have a significant role in both the transmission and expression of these genes to diabetic patients. *A. baumannii* associated with carbapenem-resistance have recently continued to increase [10, 19 21,27,29]. Our study found these novel genes as well as the $bla_{OXA\text{-}89}$ gene and $bla_{OXA\text{-}66}$ gene are associated with *A. baumannii* infections in diabetics in Saudi Arabia (prevalence of 45%, 25% and 15% respectively). All isolates containing novel $bla_{OXA\text{-}51\text{-}like}$ genes were sensitive to imipenem and meropenem except four isolates (15%) which had low-level resistance to meropenem. PFGE molecular typing revealed five clones of OXA-51-like containing strains and the novel OXA-131 β-lactamase was predominant in *A. baumannii* from patients with diabetes mellitus in Saudi Arabian hospitals.

CONCLUSION

Our study suggests that carbapenem resistance in *A. baumannii* may emerge in diabetics in regions that have, up to now, managed to avoid the problem. The dissemination of the strains of *A. baumannii* carrying the OXA-131 β-lactamase, with the insertion sequence IS*Aba1* upstream of the encoding gene, may threaten diabetics and may increase the risk due to the upregulation of $bla_{OXA\text{-}131}$ gene expression. Our finding that diabetics are infected with specific clones of *A. baumannii*, which are different from those previously found in the general population, indicates that diabetics may be vulnerable to certain strains of *A. baumannii*. Moreover, the clonal spread of such strains in patients with diabetes mellitus may prove a bigger problem to solve than in non-diabetic patients, and further highlights the need for new class D carbapenemase inhibitors.

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Appendix C.

Abstracts of conference presentations.

The Linkage of Specific *bla*$_{OXA-51}$-like Genes in *Acinetobacter baumannii* to Common Multi-resistant Clones.

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Background:
Multidrug-resistant *Acinetobacter baumannii* is a major nosocomial pathogen that has become endemic in many areas. The intrinsic *bla*$_{OXA-51}$-like genes of this species have been proposed as a genotypic marker for rapid identification, however there are many variants in the *bla*$_{OXA-51}$-like gene group. The aim of this study was to investigate whether specific *bla*$_{OXA-51}$-like genes are associated with specific genotypes.

Methods:
A panel of 20 geographically and temporally diverse *A. baumannii* isolates was screened for *bla*$_{OXA-23}$-like, *bla*$_{OXA-40}$-like, *bla*$_{OXA-58}$-like, and *bla*$_{OXA-51}$-like genes, IS*Aba1*, IS*Aba2* and IS*Aba3* by PCR amplification. *bla*$_{OXA-51}$-like products were sequenced and identified using BLAST and MultAlin software. MICs were carried out following standard British Society for Antimicrobial Chemotherapy procedure. Pulsed-field gel electrophoresis (PFGE) typing was performed using *ApaI* restriction endonuclease and the similarity of the banding patterns were analysed by BioNumerics version 4 software.

Results:
The majority of these diverse isolates fall into three major groups based on the carriage of *bla*$_{OXA-51}$-like enzymes. Ten isolates contain *bla*$_{OXA-66}$, three contain *bla*$_{OXA-69}$, and four contain *bla*$_{OXA-71}$, with the remaining three isolates each containing a unique enzyme. Each enzyme was associated with distinct genotype clusters. The *bla*$_{OXA-66}$ and *bla*$_{OXA-69}$ groups contain IS*Aba1*, but not the *bla*$_{OXA-71}$ group. *bla*$_{OXA-58}$, IS*Aba2* and IS*Aba3* are associated with one another and are spread across the groups. All isolates were resistant to ceftazidime, one isolate was resistant to imipenem and meropenem, two isolates had intermediate resistance to imipenem and meropenem, and two isolates had intermediate resistance to imipenem only.

Conclusions:
These data indicate that individual *bla*$_{OXA-51}$-like genes are associated with specific clusters that have disseminated across Europe.

The OXA-51-like Enzymes of *Acinetobacter baumannii*: Markers of Success?

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Objectives:

Acinetobacter baumannii has become a prevalent nosocomial pathogen due to the spread of major epidemic lineages. The diversity of the intrinsic $bla_{\text{OXA-51-like}}$ genes may provide insights into the evolution of this species. This study aimed to examine the relationship between the sequences of the OXA-51-like enzyme family and the properties of a collection of *A. baumannii* isolates.

Methods:

Sixty *A. baumannii* isolates had their $bla_{\text{OXA-51-like}}$ gene amplified by PCR using external primers, and the products sequenced and identified using BLAST and MultAlin software. The external primers allowed the simultaneous identification of IS*Aba1* upstream of the $bla_{\text{OXA-51-like}}$ genes. MICs for imipenem and meropenem were determined according to British Society for Antimicrobial Chemotherapy (BSAC) guidelines. Isolates were assigned to sequence groups (SGs) based on PCR amplification of fragments of their $bla_{\text{OXA-51-like}}$, *ompA* and *csuE* genes. All publicly available OXA-51-like amino-acid sequences were retrieved and used to construct a linkage map showing the relationships of the enzymes to one another.

Results:

The linkage map revealed that some of the enzymes form closely related clusters while others are less closely related. The largest number of isolates, including European clone II, contained enzymes in the OXA-66 cluster, and were assigned to SG1. The second largest group of isolates, including European clone I, contained enzymes in the OXA-69 cluster, and were assigned to SG2. The third largest isolate group, including European clone III, were assigned to SG3 and contained an OXA-71 enzyme. Eight isolates contained enzymes not found in a major cluster, or could not be assigned to a SG. IS*Aba1* was found upstream of the $bla_{\text{OXA-51-like}}$ gene in ten isolates, but was only associated with enzymes on branch tips in the OXA-66 and OXA-69 clusters. MICs for imipenem and meropenem ranged from 0.06 and 0.12 mg/L respectively up to >128 mg/L for both antibiotics. Isolates with IS*Aba1* upstream of the $bla_{\text{OXA-51-like}}$ gene tended to have MICs towards the higher end of this range, from 0.5 and 2 mg/L up to 8 and 32 mg/L.

Conclusion:

SG1 and SG2 represent the most prevalent epidemic lineages of *A. baumannii* and encode specific sub-sets of OXA-51-like enzymes. SG3 is not as prevalent, but is also associated with a specific OXA-51-like enzyme. A minority of isolates cannot be grouped using this typing scheme. Evolution of the OXA-51-like enzymes appears to be occurring in real time.

Typing and *bla*$_{\text{OXA-51-like}}$ genes of *Acinetobacter baumannii*.

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Background:
The degree of genetic flux within *Acinetobacter baumannii* may cause problems for genome-wide typing techniques. This study aimed to compare pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) schemes, and to evaluate the intrinsic *bla*$_{\text{OXA-51-like}}$ gene as a typing scheme candidate.

Methods:
A collection of 28 epidemiologically unrelated *A. baumannii* isolates were analysed by PFGE with restriction endonuclease *Apa1*, and results analysed using BioNumerics version 4 software. The *gltA*, *rpoD*, *gdhB*, *gyrB*, *recA*, *cpn60*, *gpi* and *bla*$_{\text{OXA-51-like}}$ genes were amplified and sequenced. Sequences were analysed using Multalin software, and isolates assigned to sequence types. Analysis of MLST profiles, *bla*$_{\text{OXA-51-like}}$ genes, and selection pressures on the MLST loci and *bla*$_{\text{OXA-51-like}}$ genes were carried out using START2 and Minitab15 software.

Results:
Twenty-three novel alleles for the MLST loci and 13 novel sequence types were identified. Isolates containing the same *bla*$_{\text{OXA-51-like}}$ allele were assigned to the same or closely related sequence types. In contrast, some isolates containing the same *bla*$_{\text{OXA-51-like}}$ gene demonstrated <50% relatedness by PFGE, and were more closely related to isolates containing different genes. Analysis of selection pressure using the Nei-Gojobori method gave d_N/d_S ratios for *gltA*, *rpoD*, *gdhB*, *gyrB*, *recA*, *cpn60*, *gpi* and *bla*$_{\text{OXA-51-like}}$ genes of 0.0000, 0.0000, 0.0615, 0.0264, 0.0624, 0.0000, 0.0347 and 0.2111 respectively. The d_N/d_S ratio for the *bla*$_{\text{OXA-51-like}}$ genes compared to the seven MLST loci was significantly higher ($p<0.001$).

Conclusions:
PFGE was less satisfactory for analysing a diverse *A. baumannii* population than MLST, and *bla*$_{\text{OXA-51-like}}$ gene content of isolates correlated more strongly with MLST data than with PFGE. The *bla*$_{\text{OXA-51-like}}$ genes are under greater positive selection pressure than the MLST loci, though the value is not so high as to preclude them from inclusion in a typing scheme.

Novel genetic context of multiple *bla*$_{\text{OXA-58-like}}$ genes in *Acinetobacter* genospecies 3.

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Objectives:

The spread of carbapenemase genes within Gram negative bacteria is of great cause for concern. In 2008, the first report of a *bla*$_{\text{OXA-58}}$ gene outwith *Acinetobacter baumannii* was reported in *Acinetobacter* genospecies 3. We had also identified a genospecies 3 isolate encoding a *bla*$_{\text{OXA-58-like}}$ gene, and the aim of this study was to examine the genetic environment of the gene to investigate the mobilisation between species.

Methods:

Restriction analysis of rRNA was used to confirm identity to the species level. Susceptibility to imipenem and meropenem was determined through the plate doubling dilution method. Screening by PCR for *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-40-like}}$ and *bla*$_{\text{OXA-58-like}}$ genes was carried out. Analysis of the genetic environment surrounding the *bla*$_{\text{OXA-58-like}}$ gene was conducted by sequencing inverse PCR products and gene-walking fragments. The structure of the surrounding sequence was confirmed using internal primers, which were also used to screen other *bla*$_{\text{OXA-58-like}}$ positive isolates in our collection.

Results:

Restriction analysis confirmed the isolate belonged to *Acinetobacter* genospecies 3. The isolate showed reduced susceptibility to imipenem and meropenem with MICs of 2 mg/L for both antibiotics. The isolate was negative for a *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-23-like}}$ or *bla*$_{\text{OXA-40-like}}$ gene, but positive for a *bla*$_{\text{OXA-58-like}}$ gene. Analysis of the genetic environment of the *bla*$_{\text{OXA-58-like}}$ gene revealed the gene was within a novel genetic structure. Upstream of the *bla*$_{\text{OXA-58-like}}$ gene was the left-hand end of an IS*Aba3* element, interrupted by an IS*Aba125* element. The elements contained putative promoter sequences. Downstream was an *araC1* and a *lysE* gene, followed by a sequence similar to the Re27 element described previously. Following this was a complex region containing the right-hand end of an IS*Aba3 tnpA* gene, interrupted by an incomplete *tnpA* gene with 99% similarity to IS*Aba3*, itself interrupted by an IS*Aba125* sequence. This region was followed by a second *bla*$_{\text{OXA-58-like}}$ gene. All other *bla*$_{\text{OXA-58-like}}$ positive isolates in our collection were negative for IS*Aba125* upstream of *bla*$_{\text{OXA-58}}$.

Conclusion:

This study is the first to report multiple copies of a *bla*$_{\text{OXA-58-like}}$ gene in an *Acinetobacter* genospecies 3 isolate, and has identified a novel structure containing two *bla*$_{\text{OXA-58-like}}$ genes and two IS*Aba125* sequences. The IS*Aba125* elements may be responsible for the duplication of the *bla*$_{\text{OXA-58-like}}$ gene.

High Prevalence of Unrelated Multidrug-resistant *Acinetobacter baumannii* Isolates in Pakistani Military Hospitals.

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Background:
Nosocomial infections with multidrug-resistant (MDR) *Acinetobacter baumannii* are an increasing problem worldwide. This study investigated the properties of *Acinetobacter* isolates from Pakistani hospitals.

Methods:
Twenty *Acinetobacter* isolates were obtained over two months from two military hospitals in Rawalpindi, Pakistan. They were genotyped by 16S-23S intergenic rRNA restriction analysis and assayed for antibiotic susceptibility. PCRs for *bla*OXA-23-like, -40-like, -58-like and -51-like genes, insertion sequences IS*Aba1*, *2* and *3*, and the *A. baumannii* ATPase gene were conducted. Isolate relatedness was determined by PFGE typing.

Results:
The isolates were identified as *A. baumannii* (17), *Acinetobacter* species 3 (1), *Acinetobacter* species 13TU (1), and 1 was an unidentified *Acinetobacter* species. *A. baumannii* isolates were resistant to between 4 and 15 of the antibiotics tested, including imipenem, piperacillin/tazobactam and cefoperazone/sulbactam. Twelve of the *A. baumannii* isolates were resistant to 5 different classes of antibiotic, 11 encoded the $bla_{\text{OXA-23}}$ gene, 12 encoded IS*Aba1*, 6 encoded IS*Aba2*, and 1 encoded IS*Aba3*. PCR products were obtained for IS*Aba1* upstream of all $bla_{\text{OXA-23}}$ genes, and downstream in 9 isolates. Twelve isolates were negative by PCR for the ATPase gene suggesting the presence of an inserted genomic island. PFGE analysis demonstrated that isolates were not clonal. More resistant isolates were associated with carriage of $bla_{\text{OXA-23}}$, $bla_{\text{OXA-66, -69}}$, or the novel $bla_{\text{OXA-144}}$, IS*Aba1* and a negative ATPase PCR result.

Conclusion:
The high prevalence of MDR *A. baumannii* infections in hospitals in Pakistan in in- and out-patients caused by non-clonal isolates combined with high carriage of mobile resistance elements and genomic islands that may facilitate the acquisition of further resistance determinants poses a serious threat to our ability to combat this organism.

Distribution of Intrinsic Plasmid *Rep* Genes and their Association with Class D OXA Carbapenemase Genes in European Isolates of *Acinetobacter baumannii*.

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Background:
Carbapenem-resistant strains of *A. baumannii* pose an increasing threat worldwide. This study examined the distribution of intrinsic plasmids in European isolates of *A. baumannii* and their potential role in the dissemination of class D carbapenemase genes.

Methods:
96 randomly selected isolates (comprising 17 different PFGE types) of *A. baumannii* from 25 hospitals in 17 European countries were screened by PCR for specific class D carbapenemase genes and 16 different groups of plasmid *rep* genes (GR1 - 16) found in *A. baumannii*, with results confirmed by DNA sequencing where necessary.

Results:
All 96 isolates contained at least one (maximum five) of the 16 groups of *rep* genes. Those detected were GR6, including the *rep*Aci6 gene (93 isolates), GR2, including *rep*Aci1 (67 isolates) and *rep*Aci2 (3 isolates), GR10 (*rep*AciX, 51 isolates), GR12 (*rep*Aci12, 10 isolates), GR16 (*rep*pAB49, 6 isolates), GR3 (*rep*Aci3, 4 isolates), GR4 (*rep*Aci4, 3 isolates), GR14 (*rep*p4AYE, 1 isolate). OXA-58-like genes (22 isolates) were associated with carriage of the *rep*Aci1, *rep*Aci3, *rep*Aci4 and *rep*AciX genes, OXA-40-like genes (6 isolates) with *rep*Aci2, *rep*AciX and *rep*p2SDF, and OXA-23-like genes (7 isolates) with *rep*Aci1 and *rep*AciX.

Conclusions:
The *rep*Aci1 and *rep*AciX genes are associated with the Re27 sequence, which seems to be a favoured insertion site for structures carrying class D OXA genes. Although class D OXA genes are also associated with other plasmids, the presence of *rep*Aci1 and *rep*AciX may indicate a particular propensity for strains to acquire such genes. Most intrinsic *Acinetobacter* plasmids are non-self-transferable, but the almost ubiquitous *rep*Aci6 gene is associated with a *tra* locus that could serve to mobilise other plasmids carrying antibiotic resistance genes such as those for class D carbapenemases.